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AN ANALYSIS OF THE TITRATION DATA OF OXYHEMOGLOBIN OF THE HORSE BY A THERMAL METHOD

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There is a great amount of data on the titration of proteins with acids and bases. Most of this has received some degree of interpretation in terms of the composition of the molecules. In a number of cases the total acid- and base-combining power has been shown to be in very close agreement with that calculated on the basis of the analytical figures for the amino acid content of the proteins in question. In at least one case, moreover, it has been possible to fit the data in a satisfactory way over the whole range by dividing the total number of acid- and base-combining groups into a relatively small number of classes and assigning to each class a suitably chosen acidity constant, and the required choice of classes and constants has not been inconsistent with the known composition of the protein.

There is another method of approach available for the analysis of the acid- and base-combining power of the proteins which so far has not been tried, but which may be expected to shed new light on the problem and to supplement the information derived in other ways. This, which we may call the thermal method, consists in determining the effect of temperature on the titration curve. Since the different acid- and base-binding groups of these polyvalent molecules are for the most part characterized by different heats of dissociation, we should expect, in accordance with the van't Hoff equation, that the effect of temperature would be different in different ranges of the titration curve, and this should make it possible to see directly in which ranges different groups are active. In this paper we describe the application of this method to the case of a well known protein, oxyhemoglobin of the horse. First we shall give the results of the titration of oxyhemoglobin in solution at three temperatures and the apparent heat of

dissociation as a function of pH calculated from these data; we shall then develop the theory underlying the method, and make use of it in the analysis of the data on oxyhemoglobin.¹

The horse hemoglobin was crystallized by the method of Ferry and Green, as described by German and Wyman (2), from concentrated cells kindly furnished by the Massachusetts Antitoxin and Vaccine Laboratory. The final crystals were dissolved in 0.3 M aqueous sodium chloride to provide the stock solution which was used for all the measurements. This solution contained, according to a Kjeldahl analysis, 12.42 gm. of nitrogen per liter, or 73.8 gm. of hemoglobin per liter, on the basis of a nitrogen factor of 5.93 (Vickery and Leavenworth (3)). At the end of the measurements the solution was found to have an oxygen-combining capacity of 9.71 volumes per cent. Assuming the molecular weight of hemoglobin to be 66,800, this indicates that only 1.9 per cent of the protein was inactive. This amount is so small that any corrections based on it are less than the experimental error, and it has been ignored in calculating the results.

Measured amounts of approximately 0.1 N HCl or NaOH were added to aliquots of stock solution, and the resulting mixture diluted with water to give the same final concentration of protein in all cases. This involved a dilution of the stock solution to 132 per cent of its original volume. The titrated aliquots were equilibrated with oxygen at a pressure of about 90 to 100 cm. of mercury in a tonometer before injection into the electrode for measurement.

The pH was determined with a glass electrode in connection with a high resistance vacuum tube bridge (1). The measurements were made at three temperatures, 6.5°, 25°, and 37.7°, controlled by water pumped from a constant temperature bath through a glass jacket surrounding the electrode. The electrode was calibrated constantly throughout the course of the measurements with various standard buffers. The composition of these buffers and the pH assigned to each for each of the three temperatures are given in Table I. The values of the pH are based on interpolations or extrapolations from data given by Clark (4) wherever the values are not given directly by him.

¹ It should be noted that in 1924 Stadie and Martin (1) made an estimate of the heat of ionization of hemoglobin as a base from the shift of the isoelectric point with temperature.

J. Wyman, Jr.

TABLE I
pH of Standard Buffers

					6.5°	25°	37.7°
10	cc. citrate*				4.92	4.98	5.03
9.0	" "	+ 1.0	cc. NaOH*		5.07	5.13	5.18
8.0	" "	+ 4.0	" "	*	5.93	6.00	6.03
0.25	" Na_2HPO_4	+ 9.75	" KH_2PO_4	†	5.31	5.28	5.26
1.0	" "	+ 9.0	" "	†	5.92	5.90	5.87
49.6	" "	+ 50.4	" "	†	6.83	6.80	6.78
84.1	" "	+ 15.9	" "	†	7.52	7.50	7.48
87.0	" "	+ 13.0	" "	†	7.64	7.60	7.58
89.4	" "	+ 10.6	" "	†	7.73	7.70	7.67
94.7	" "	+ 5.3	" "	†	8.05	8.01	7.98
10	" borate‡				9.33	9.19	9.10

* Clark (4), Table 42.

† Clark (4), Tables 41 and 43.

‡ Clark (4), Table 43.

§ Clark (4), Table 38.

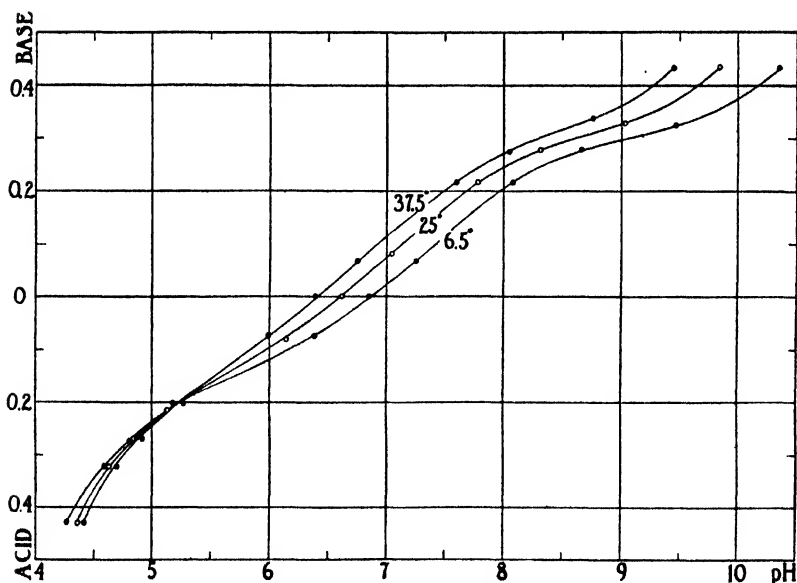


FIG. 1. Titration curves of oxyhemoglobin at three temperatures. Acid and base are expressed in milliequivalents per gm. of hemoglobin.

The titration curves for each of the three temperatures are shown graphically in Fig. 1. In Fig. 1 the ordinates give milliequivalents of acid or base per gm. of hemoglobin, which, if desired, may be converted into equivalents per mole simply by multiplying by 66.8 on the assumption of a molecular weight of 66,800. The results at 25° are in good agreement with other data and it is unnecessary to tabulate them numerically.

The point in which we are primarily interested here is a second order effect; namely, the difference of pH between the three curves for given values of the ordinates. It is this quantity which determines what we shall call the apparent heat of dissociation, Q' , and define, by analogy with the case of a weak monobasic acid, by the equation

$$(1) \quad Q' = -2.303 RT^2 \left(\frac{\partial \text{pH}}{\partial T} \right)_B$$

In this equation and subsequently, R denotes the gas constant, T the absolute temperature, and B the total amount of base present. The justification of this definition is given below. In Fig. 2 we have plotted values of this quantity expressed in calories as a function of pH over the entire range covered by the data. These values were actually calculated from the pH values taken from the smooth curves of Fig. 1 at various convenient values of the ordinate by the expression

$$(2) \quad -4.579 T_1 T_2 \frac{\text{pH}_2 - \text{pH}_1}{T_2 - T_1}$$

in which subscripts 1 and 2 refer to the two different temperatures in question. In Fig. 2 each value so calculated is plotted against the corresponding mean pH; *i.e.*, $(\text{pH}_2 + \text{pH}_1)/2$.

Let us now consider first very briefly the case of a simple acid SH which dissociates to give the products S^- and H^+ in accordance with the equation

$$(3) \quad \frac{a_{\text{S}} a_{\text{H}^+}}{a_{\text{SH}}} = k$$

where a denotes activity and k is the thermodynamic equilibrium constant. Q , the heat of dissociation per mole,² is given by the

² This is the increase of total heat; *i.e.*, the heat absorbed as a result of the dissociation.

van't Hoff equation

$$(4) \quad Q = RT^2 \frac{\partial \ln k}{\partial T}$$

Actually, what is more readily determined from the titration data is not the thermodynamic constant k but the acidity constant k' defined as

$$(5) \quad \frac{C_{S^-}}{C_{SH}} a_{H^+} = k' = k \frac{f_{SH}}{f_{S^-}}$$

in which C denotes concentration, and f activity coefficient. The van't Hoff equation, expressed in terms of k' , is

$$(6) \quad Q = RT^2 \frac{\partial}{\partial T} \left(\ln k' + \ln \frac{f_{S^-}}{f_{SH}} \right)$$

The expression $RT^2(\partial \ln k' / \partial T)$ gives what we call the apparent heat of dissociation Q' . This may be calculated from the titra-

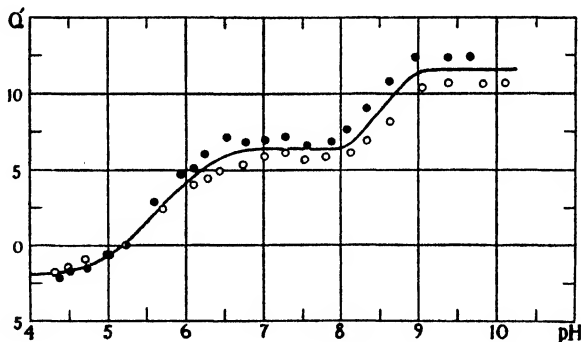


FIG. 2. Apparent heat of dissociation of oxyhemoglobin as a function of pH. ● = values for the 25–37.7° interval; ○ = values for the 6.5–25° interval.

tion data at once without any knowledge of activity coefficients from the equation

$$(7) \quad Q' = RT^2 \frac{\partial \ln k'}{\partial T}$$

Q' is of course not the same as the true heat of dissociation Q , but in general it is very close to it, since $\ln (f_{S^-}/f_{SH})$ varies but little

with temperature regardless of its absolute magnitude. The fact that Q' involves only the temperature derivative of f_{S^-}/f_{SH} and not this quantity itself is of considerable importance for the more complex case about to be considered, since it shows that even very great modifications in the residue attached to a given dissociating group can have little or no effect on the apparent heat of dissociation in so far as they lead only to changes in f_{SH} and f_{S^-} , however great. Provided the acid is sufficiently weak in relation to its concentration and the pH of the solution to allow us to make the usual approximation of setting C_{S^-} equal to the amount of base added,³ the apparent heat of dissociation may be simply reckoned from the change of pH with temperature of a partially titrated solution containing a fixed amount of base, by the following equation, which results from Equations 5 and 7

$$(8) \quad Q' = -RT^2 \left(\frac{\partial \text{pH}}{\partial T} \right)_E$$

This method of reckoning Q' is the only one practicable in the more complex case which we will now consider, that of a polyvalent molecule, like a protein, containing n acid or basic groups. We shall treat all these groups as acid groups and express their dissociation in terms of n acidity constants k . For convenience of notation in what follows we shall omit the prime marks when referring to the acidity constants and apparent heats of dissociation and shall use H to refer to the activity of the hydrogen ions, omitting the symbols a and $+$. We shall assume that the n groups are independent, so that the dissociation of each is unaffected by that of the others. As a result of the independent dissociation of these n groups there will be $n + 1$ classes of ions, to which we shall refer by subscripts 0, 1, . . . n . Each class is characterized by the total number of hydrogen ions dissociated, or, what amounts to the same thing, by the number of equivalents

³ The error involved in this approximation is

$$\frac{C_{S^-} - C_B}{C_{S^-}} = \frac{C_{H^+}(C_{SH} + k')}{k'(C_{SH} + C_{S^-})}$$

provided we assume the base to be completely dissociated, neglect the dissociation of water, and take $C_{H^+} = a_{H^+}$.

of base bound, per mole, and this number is the same as the subscript. These $n + 1$ classes are all in equilibrium with one another, and the concentration C_{Si} of ions in the i 'th class may be expressed in terms of the concentration C_{S_0} of ions of the 0'th class as follows:

$$(9) \quad \frac{C_{Si}}{C_{S_0}} = \frac{K_i}{H^i}$$

Each of the n constants K depends on the n individual k 's and K_i is the sum of all the $n!/(n-i)!i!$ different products of i k 's chosen from the total number of n k 's. The total concentration of all the ions of all classes is

$$(10) \quad C_S \left(1 + \frac{K_1}{H} + \dots \frac{K_n}{H^n} \right) = C_S A$$

and the total amount of base bound is

$$(11) \quad C_S \left(\frac{K_1}{H} + \frac{2K_2}{H^2} + \dots \frac{nK_n}{H^n} \right) = C_S G$$

We have introduced the symbols A and G for convenience to refer to the two expressions in parentheses. The amount of base bound per mole of material is

$$(12) \quad b = G/A$$

A formal differentiation of b in terms of $\ln H$ and $\ln K_1, \dots \ln K_n$ gives

$$(13) \quad db = \left(\frac{\partial b}{\partial \ln H} \right)_{K_i} d \ln H + \sum_{i=1}^n \left(\frac{\partial b}{\partial \ln K_i} \right)_{H, K_j} d \ln K_i$$

If b is constant and $\ln H$ and $\ln K_i \dots$ are expressed as functions of the temperature, this becomes, after multiplication by RT^2 ,

$$(14) \quad -RT^2 \left(\frac{\partial \ln H}{\partial T} \right)_b = \sum_{i=1}^n \left(\frac{\partial b}{\partial \ln K_i} \right)_H \frac{Q_i}{(\partial b / \partial \ln H)_{K_i}}$$

in which

$$(15) \quad Q_i = RT^2 \frac{\partial \ln K_i}{\partial T}$$

If we make use of Equations 10, 11, and 12 and remember that $\partial \ln x / \partial x = 1/x$, Equation 14 becomes

$$(16) \quad RT^2 \left(\frac{\partial \ln H}{\partial T} \right)_b = \frac{A \sum_{i=1}^n \frac{iK_i Q_i}{H^i} - G \sum_{i=1}^n \frac{K_i}{H^i} Q_i}{A \sum_{i=1}^n \frac{i^2 K_i}{H^i} - G^2}$$

It follows from the structure of K_i that Q_i consists of $n!/i!(n-i)!$ terms all constructed in the same way, of which the first is

$$k_1 k_2 \cdots k_i (q_1 + q_2 + \cdots q_i) / K_i$$

Each q is the apparent heat of dissociation of an individual group; e.g., $q_1 = RT^2 \partial \ln k_1 / \partial T$. Q_i is the apparent heat of dissociation for the total step from class 0 to class i : $S_0 \rightarrow S_i + i$ hydrogen ions. $Q' = RT^2 (\partial \ln H / \partial T)_b$ is the apparent heat of dissociation of the molecule as a whole and may be determined from the experimental data by Equation 1, as was done in constructing Fig. 2.

When the q 's are the same for all the groups and equal to q , Q' calculated from Equation 16 reduces to q , as we know it must. When the q 's are different, but the k 's are all the same, it follows from the same equation that Q' is independent of pH and equal to the arithmetic mean of all the q 's. If the groups fall into two equal classes, each characterized by a given q and k , then Q' is the same as for a simple dibasic acid with one group having one k and q , the other group having the other k and q . Equation 16 shows how in any given case Q' varies as the pH is changed. The sharpness of the transition of Q' from one level to the next and the separation of the levels depend on the spacing of the successive pK 's as well as on the corresponding q 's and increase as the spacing increases.

The behavior of Q' in a number of special cases is illustrated in Figs. 3 and 4. Fig. 3 is for a molecule containing two groups having the constants pk_1 and $pk_2 > pk_1$ and heats q_1 and $q_2 > q_1$. The ordinate gives $(Q' - q_1)/(q_2 - q_1)$; the abscissa gives $pH - (pk_1 + pk_2)/2$. The curves are all symmetrical and in each case the value of the upper asymptote is given. The positions of the pk 's are shown by the circles.⁴ It may be seen that when the

⁴ I.e., values of the abscissa for which $pH = pk_1$ or $pH = pk_2$.

separation of the pk's is about 1.5 units or more the case is not very different from that in which the pk's are infinitely separated.

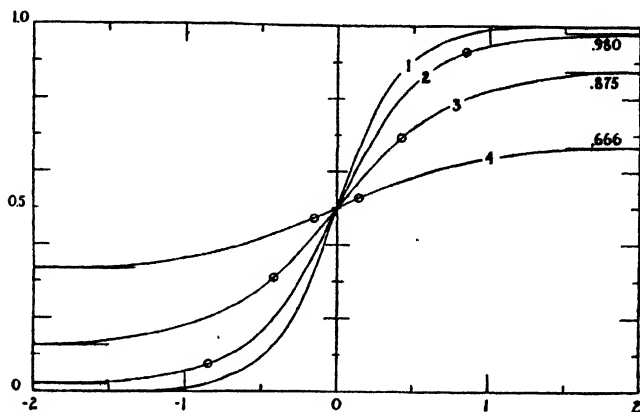


FIG. 3. The ordinates give $(Q' - q_1)/(q_2 - q_1)$; the abscissas $\text{pH} - (\text{pk}_1 + \text{pk}_2)/2$. Curve 1, $\text{pk}_2 - \text{pk}_1 \rightarrow \infty$; Curve 2, $\text{pk}_2 - \text{pk}_1 = 1.690$; Curve 3, $\text{pk}_2 - \text{pk}_1 = 0.845$; Curve 4, $\text{pk}_2 - \text{pk}_1 = 0.301$.

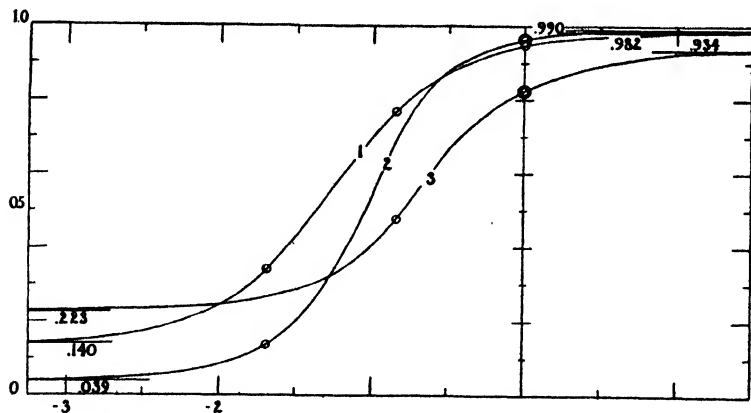


FIG. 4. The ordinates give $(Q' - q_1)/(q_2 - q_1)$; the abscissas $\text{pH} - \text{pk}_1$. Curve 1, $\text{pk}_2 - \text{pk}_1 = \text{pk}_2 - \text{pk}_1 = 0.845$; Curve 2, $\text{pk}_2 = \text{pk}_1$, $\text{pk}_2 - \text{pk}_1 = 1.690$; Curve 3, $\text{pk}_2 = \text{pk}_1$, $\text{pk}_2 - \text{pk}_1 = 0.845$.

Fig. 4 illustrates three cases of a molecule containing three groups characterized by pk_1 and q_1 , $\text{pk}_2 > \text{pk}_1$ and $q_2 > q_1$, and $\text{pk}_3 \geq \text{pk}_2$ and $q_3 = q_2$. The ordinates give $(Q' - q_1)/(q_2 - q_1)$ and

the abscissas $\text{pH} - \text{pk}_3$. Here again the positions of the pk 's are indicated with circles, and the values of the upper and lower asymptotes are given.

In the light of this analysis it appears from Fig. 2 that over the range from pH 4 to pH 10 the dissociating groups of oxyhemoglobin fall into three classes on the basis of the heat of dissociation. Members of Class 1 have an average apparent heat of dissociation of -2000 or -3000 calories, and are active in the acid range of the titration. Members of Class 2 have an average apparent heat of dissociation of about $+6200$ calories and are active in the middle range. Members of Class 3 have an average apparent heat of dissociation of $+11,500$ calories and are active in the alkaline range of the titration. These three classes correspond to the three plateaus of Fig. 2, separated by two transition regions whose mid-points lie close to pH 5.5 and 8.5 respectively. The right-hand transition is complete in about 1 pH unit and this rather sudden transition suggests a wide separation, 2 units or more, between the pk of the most alkaline group of Class 2 and that of the most acid group of Class 3, which must be, respectively, not more than about 7.5 and not less than about 9.5. With a separation of the nearest groups of the two classes as great as this the effect of other groups further to the right and left is negligible.

In contrast to this, the left-hand transition in Fig. 2 occupies at least 2 pH units. This suggests that the separation of the pk of the last group of Class 1 and that of the first groups of Class 2 is much less than 2 units and that other groups to the right and left have an effect. This situation when the groups are closely spaced is more complicated. Fig. 4 gives some idea of what may be expected. Curve 1, which represents the case where the three pk 's are equally spaced and separated by 0.85 unit, is the one in which the transition is most flattened and prolonged. The addition of other groups to the right and left would serve to extend the transition slightly more and to shift the asymptotes toward $+1$ and -1 , and with this modification Curve 1 is probably fairly representative of the acid transition of Fig. 2. We may conclude, therefore, that the pk of the most alkaline group of Class 1 is about 5 and that of the most acid groups of Class 2 about 6.

Let us consider the amount of base bound by the groups of Class 2, having a heat of dissociation of 6200 calories. If we recall that more than 80 per cent of the titration of any acid group is accomplished within a space of 2 pH units centered about the pK value, we see that there can be very little overlapping as regards the base bound by these groups and those of Class 3. The latter do not begin to bind any considerable amount of base up to about pH 8.5 or more. In the range of the left-hand transition of Fig. 2 we may expect an appreciable amount of overlapping as regards base bound by groups belonging to Classes 1 and 2. Consideration shows, however, that owing to the roughly symmetrical way in which the overlapping occurs we obtain approximately the correct result by attributing all the base bound on the alkaline side of the mid-point of the left-hand transition region of Fig. 2, *i.e.* pH 5.5 or 5.6, to the groups of Class 2 and all the base bound on the acid side of this point to groups of Class 1. The difference between the total amount of base bound per gm. of protein at 25° between pH 8.5 and 5.5 read from the middle curve of Fig. 1 is 0.46 equivalent. This corresponds to 31 equivalents per mole. This is almost the same as the analytical figure for the number of histidine units per mole; namely, 33 (3, 5). Now the characteristic heat of dissociation of the imidazole group of histidine is known to be 6200 or 6900 calories (6, 7). There can be little doubt therefore that Class 2 consists of the imidazole groups of the histidine units. Since the pH range covered by groups of Class 2 includes the range where the base-binding power of hemoglobin is affected by oxygenation (2), it must be certain of these groups which lie close to the oxygen-combining centers and are affected by the introduction of oxygen into the molecule.

On the other hand, the groups of Class 1, the acid class, are certainly carboxyl groups. These are known to have a very small heat of dissociation, sometimes positive, sometimes negative, but never more than ± 2000 or 3000 calories (6) and this agrees well with the observed heats.

The remaining groups to be considered are the guanido groups of the 13 to 16 arginine units (4, 5), the amino groups of the 37 to 44 lysine units (4, 5), the hydroxyl groups of the 12 tyrosine units (8), and the sulfhydryl groups of the somewhat uncertain number of cysteine units, given by chemical analysis of the hemoglobin

molecule. Data are lacking on the heat of dissociation of the sulfhydryl group, but that of the hydroxyl group in dihalogenated tyrosines is 800 to 900 (7), that of the guanido group of arginine is 12,400 (7), and that of the amino group is between 9000 and 12,000 (7). All these groups dissociate at strongly alkaline reactions, but our results indicate that the strongly alkaline groups of Class 3 must be either the amino groups of lysine or the guanido groups of arginine.

This analysis is in good accord with the recent results of Cohn and others (9) involving the titration of carboxyhemoglobin, which is believed in this respect to be like oxyhemoglobin; in the absence of salt. They have shown that between pH 1.5 and 13 the curve involves in all the dissociation of 261×10^{-5} equivalent of hydrogen ions per gm. or 174 equivalents per mole of hemoglobin, and they show that the data may be fitted within the accuracy of the experiments by assuming the presence in each molecule of fourteen groups of $pK' = 11.6$, forty of $pK' 10.8$, twenty of $pK' 7.5$, thirteen of $pK' 5.7$, four of $pK' 4.8$, and 83 of $pK' 3.7$ or 4.0 . They suggest, as our results confirm, that the thirty-three groups of $pK' 5.7$ or 7.5 are the imidazole groups of histidine. They also suggest that the forty groups of $pK' = 10.8$ are the amino groups of lysine, which accords with the heats that we have observed.

SUMMARY

The apparent heat of dissociation of a polyvalent substance like a protein is defined in terms of the effect of temperature on the titration curve. An analysis is given showing how this apparent heat of dissociation is related to the heats of dissociation of the individual groups of the molecule. From the observed variation of the apparent heat of dissociation with pH and a knowledge of the characteristic heats of dissociation of the individual groups it is possible to decide which groups are active in different ranges of the titration curve.

The apparent heat of dissociation of oxyhemoglobin of the horse has been determined between pH 4 and 10. The data indicate that the groups active acid to pH ~ 5.5 are carboxyl groups, that those active between pH ~ 5.5 and pH ~ 8.5 are the imidazole groups of histidine residues, and that those active alkaline

to pH \sim 8.5 are either amino groups or the guanido groups of arginine residues. From this it follows that the groups affected by oxygenation of hemoglobin are the imidazole groups of a certain number of histidine residues.

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DETERMINATION OF CHOLESTEROL

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The choice of a method for the determination of cholesterol in biological samples is determined by two factors: (a) the time available for the analyses and (b) the degree of accuracy desired. Because of their extreme simplicity, the methods of Bloor (1) for total cholesterol and of Bloor and Knudson (2) for ester cholesterol are the methods of choice for many routine determinations. These methods make use of the Liebermann-Burchard color reaction applied to the blood lipids without saponification. In general, the results are 10 to 20 per cent higher than by the digitonin precipitation methods and are sometimes affected by other colors in or produced by the blood extracts. For precise analytical results, it is necessary to use more exacting procedures. The following method has been in use in this laboratory for 2 years and, since all of the several steps are in common use in fat analyses, it is relatively simple in technique. No tricky procedures are involved in the method, nor is any special apparatus other than an ordinary colorimeter required.

In brief, the method consists of the formation of insoluble cholesterol digitonide, either from the original or from the saponified sample. The digitonide, after purification with petroleum ether, is decomposed by boiling in benzene and the free sterols isolated by petroleum ether extraction. This material is assayed by the Liebermann-Burchard reaction.

Methods

Preparation of Extract for Analysis

Alcohol-ether extracts of the blood or tissue to be analyzed are made according to the classical methods of Bloor (3). Aliquots of

the filtered extract are chosen so as to contain approximately 0.5 mg. of cholesterol. Since the method is intended to be used as an integral part of the general Bloor technique for differential lipid analysis, the reader is referred to his publications (3, 4) for the details of the extraction of the sample.

Total Cholesterol—For the determination of total cholesterol, it is necessary to saponify the bound cholesterol before the precipitation with digitonin. Evaporate a suitable aliquot of the sample to a paste in a 50 cc. Erlenmeyer flask on the steam bath. Mix 10 cc. of 1:1 alcohol-ether and 0.3 cc. of potassium hydroxide solution¹ and add to the sample. Bring to a boil and set on the back of the steam bath or in the incubator at 37° for half an hour. At the end of this time, neutralize the sample by adding 1.0 cc. of dilute hydrochloric acid² and evaporate to a paste on the steam bath. While the sample is still warm, add 10 cc. of petroleum ether and boil on the steam bath until about half of the petroleum ether has evaporated. Carefully decant the petroleum ether into a 15 cc. centrifuge tube without letting any of the aqueous layer pass over. Extract the residue a total of four times and concentrate the sample to 0.5 cc. with the aid of a boiling rod.³

Free Cholesterol—For the determination of free cholesterol, it is necessary to have a phospholipid-free sample, since, as has been shown by McEwen (5), digitonin causes the precipitation of phospholipid as well as sterols. In most cases, the acetone-soluble fraction from the phospholipid precipitation is available for this analysis. If only free cholesterol is to be determined in the sample, the phospholipid is precipitated according to the method of Bloor (3) and the acetone-soluble fraction concentrated to 0.5 cc. with the aid of a boiling rod.

¹ Prepared by dissolving 35 gm. of 85 per cent potassium hydroxide and making to 100 cc.

² Prepared so that 1.0 cc. is sufficient to make 0.3 cc. of the potassium hydroxide solution definitely acid to litmus.

³ Pull out a 10 inch length of 3 mm. glass rod so that the center is about 0.5 mm. in diameter. Break in the center and heat the narrowed end in the flame until a glass drop is formed which is about the same size as the original rod. While still hot, make a small hole in the bottom of the glass drop by pressing onto an inverted thumb-tack. This rod will permit solutions in centrifuge tubes to boil rapidly without bumping.

Isolation of Cholesterol

Add 4.0 cc. of the digitonin solution⁴ to the prepared sample in the centrifuge tube. Evaporate to dryness in a beaker of hot water on a hot-plate and dissolve the contaminating substances by boiling the precipitate in 12 cc. of petroleum ether. Remove the boiling rod and centrifuge the tube for 3 to 4 minutes. The flaky precipitate is usually well packed in this length of time and the supernatant liquid can be discarded. Repeat this extraction twice. If desirable, the decantates from the free cholesterol samples can be saved for saponification and determination of ester cholesterol.

Replace the boiling rod in the centrifuge tube, add 7.0 cc. of benzene, and boil evenly and slowly for a total of 45 minutes. Replace the benzene as needed in the tube in order to keep the volume above 5 cc. At the end of the heating period, concentrate the benzene to 3 cc. and add petroleum ether to bring to 12 cc. while still warm. On stirring, the digitonin will be precipitated and can be centrifuged out. The technique of extraction is the same as before, except that this time the purified cholesterol is contained in the petroleum ether washings. To remove all of the cholesterol, three extractions are recommended. Combine the decantates in a 50 cc. Erlenmeyer flask and evaporate to dryness on the steam bath. Remove the last traces of solvent with a gentle stream of air. The sample is now ready for the actual determination of the cholesterol content.

Colorimetric Assay

Pipette exactly 5.0 cc. of chloroform into the sample, carefully rinsing down the sides of the Erlenmeyer flask. Decant this solution as completely as possible into a 10 cc. glass-stoppered graduated cylinder. Since the concentration and not the total amount is the measured factor in colorimetric determinations, it is necessary only to have the sample made up to a known volume. Add 1.0 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid to both the sample and the standard solution. The

⁴ Make a 0.2 per cent solution of digitonin in 95 per cent alcohol by warming on the steam bath until the solution is clear.

standard solution contains 0.500 mg. of cholesterol in 5.0 cc. of chloroform. Mix the contents of both cylinders by inversion and set in a water bath at about 23° for 15 minutes under moderate light conditions. With a red Wratten color filter No. 29 placed over the eyepiece of the colorimeter, compare the sample with the standard.

DISCUSSION

Decomposition of Cholesterol Digitonide—Benzene has been found to be the most satisfactory reagent for the decomposition of the purified cholesterol digitonide. Xylene and pyridine have long been used to recover the expensive digitonin from samples, but these compounds, as well as the glacial acetic acid used by Yasuda (6) and by Smith and Marble (7), are high boiling and therefore difficult to remove from the purified cholesterol. In addition, the use of glacial acetic acid causes the formation of cholesterol acetate (7), which would then have to be saponified, since ester cholesterol cannot be quantitatively compared to free cholesterol in the colorimeter, as is shown below.

Several compounds were tested as decomposing agents in addition to those already named. Among these were a solution of sodium acetate in alcohol (recommended by Lifschütz (8)), carbon tetrachloride, toluene, alcohol, and benzene. Benzene was found to be the most practical. Apparently the decomposition is brought about by the heating process, since more time is required for the quantitative liberation of the cholesterol when lower boiling reagents are used.

Purification of Cholesterol before Assay—The discovery by Gardner and Williams in 1921 (9) that esterified cholesterol reacts with the Liebermann-Burchard reagents at a greater rate than free cholesterol has not been given the consideration that it deserves. This discrepancy is vitally important, as is shown in Fig. 1. These values were obtained by comparing the apparent cholesterol content of the sample after the color was allowed to develop for varying lengths of time, but with a fresh cholesterol standard for each time interval. The same amount of cholesterol (0.500 mg.) was present in each sample. The color in each standard was allowed to develop for 15 minutes at 23° after the addition of the color reagents. All data represent duplicate determinations, with the exception of the 15 minute values which

were repeatedly checked. The apparent recovery of the cholesterol when present as the palmitate is about 130 per cent.

The original notation by Gardner and Williams (9) states that, "In the case of cholesteryl stearate the figures were slightly higher than truth." No data are given for the stearate, but values for the benzoate and acetate are calculated to be 104.6 and 111.0 per cent, respectively.

This phenomenon has been fully confirmed by Reinhold (10) who was apparently unaware of Gardner and Williams' findings. In 1934, he reported that cholesterol palmitate gave about 120 per cent apparent recovery, his own specific directions for the

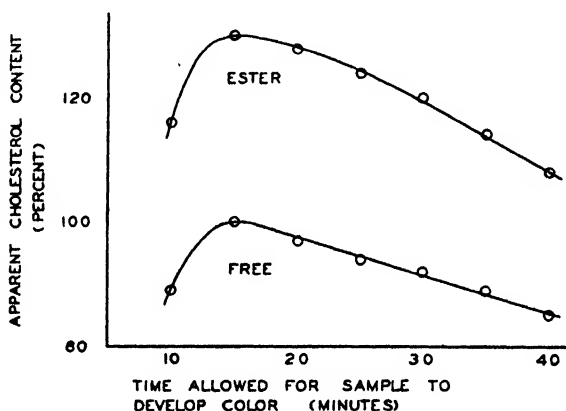


FIG. 1. Rate of color development

development of the color being used. If the ester had been previously hydrolyzed, this enhancement effect was lost. Yasuda (11) reported this same phenomenon, while apparently unaware of both Gardner and Williams' and of Reinhold's work. Yasuda tested the chromogenic properties of cholesterol palmitate, stearate, oleate, and a sample of cholesterol esters prepared from pig serum. The enhancement effects were 24.5, 25.2, 27.6, and 23.0 per cent, respectively. They probably account for the higher values obtained by Bloor ((1) p. 231) on unsaponified blood extracts and may account for the fact that his ester percentages are lower than those obtained by Sperry (12).

Because of this effect, it seems quite conclusive that for precise

Cholesterol Determination

TABLE I
Recovery Analyses on Known Samples of Cholesterol Palmitate and Free Cholesterol

Total cholesterol recovery*		Free cholesterol recovery†	
mg.	per cent	mg.	per cent
0.882	99.5	0.506	101.2
0.862	97.5	0.513	102.6
0.882	99.5	0.524	104.8
0.873	98.5	0.506	101.2
0.882	99.5	0.503	100.6
0.882	99.5	0.510	102.0
0.882	99.5	0.506	101.2
0.862	97.5	0.510	102.0
0.882	99.5		
0.852	96.0		
0.843	95.5		
Average.	98.5		101.9
" deviation	1.3		0.9

* Each sample contained 0.886 mg. of cholesterol as the palmitate.

† Each sample contained 0.500 mg. of free cholesterol.

TABLE II
Cholesterol Analyses on Alcohol-Ether Extract of Plasma
 The values are given in mg. per cent.

Sample No.	Total cholesterol		Free cholesterol	
	Original extract	Fortified extract*	Original extract	Fortified extract*
1	145.8	196.0	48.8	99.0
2	149.0	193.2	49.2	98.4
3	143.6	192.0	49.0	96.6
4	147.3	197.6	49.8	97.0
5	145.0	192.0	49.2	97.6
6	142.4	194.0	49.8	97.6
7	143.0	195.2	48.8	98.0
8	145.0	193.2	49.0	99.0
Average	145.7	194.0	49.2	97.9
" deviation	1.7	1.6	0.3*	0.7

* Original extract fortified by the addition of the equivalent of 50 mg. per cent of free cholesterol.

work the Liebermann-Burchard reaction must be carried out with the cholesterol in the free form. Furthermore, since other lipids give varying degrees of color with these reagents, the cholesterol must be fairly free of contaminating substances. In the method reported here, the color is developed on a sample consisting of the digitonin-precipitable sterols freed from other lipids as well as from the digitonin.

Reproducibility of Method—Table I shows the results obtained with cholesterol palmitate for the total, and pure cholesterol for the free cholesterol determinations. It may be seen that the recovery is within the experimental error inherent in colorimetric readings.

In Table II are reported the analyses on an alcohol-ether extract of plasma. 50 cc. of plasma were extracted with 20 volumes

TABLE III
Summary of Recovery Experiments

Cholesterol (8 determinations on each)	Original extract	Fortified extract			
		Calculated	Recovery		Average deviation
	mg. per cc.	mg. per cc.	mg. per cc.	per cent	per cent
Total.....	0.07290	0.09790	0.09700	99.0	4.6
Free.....	0.02460	0.04960	0.04895	98.7	1.5

of 3:1 alcohol-ether, filtered, and aliquots taken for the analyses. This extraction was duplicated, 50 cc. of the same plasma being used, but 25 mg. of free cholesterol were added to the alcohol-ether before filtering, so that recovery in a biological sample could be tested. Recoveries, with average deviation values, are given in Table III.

All of the several steps in the procedure have been examined to show that quantitative recoveries are possible. Sufficient data are recorded in the literature to show that the original extraction of the material to be analyzed is satisfactory (3, 4). The conditions for the precipitation of the digitonide are identical with those used by Bloor and Knudson (2), which time has shown to be satisfactory. The recovery analyses on pure cholesterol show that the decomposition with benzene is quantitative (and the Liebermann-Burchard reaction is classical).

SUMMARY

A micromethod for the quantitative isolation of the digitonin-precipitable sterols from biological samples has been devised. Colorimetric analysis of this material gives an accurate picture of the true cholesterol content of the sample, because of the absence of the naturally occurring contaminants which usually affect the Liebermann-Burchard reaction. Both free and total cholesterol analyses can be made on the same sample.

The author wishes to acknowledge the helpful advice of Dr. W. R. Bloor throughout the course of this investigation.

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ADDITIONAL NUTRITIONAL FACTORS REQUIRED BY THE RAT*

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Purified rations have been used in vitamin studies with rats for many years but it is only very recently that it has been possible to supply an appreciable number of the vitamins in pure form. Three members of the vitamin B complex are now available commercially, and vitamin B₆ is available on an experimental basis (Lepkovsky (1), Keresztesy and Stevens (2), György (3), and Kuhn and Wendt (4)). When rats are placed on purified diets low in the vitamin B complex but supplemented with thiamine, riboflavin, nicotinic acid, and concentrates or crystals of vitamin B₆, very poor growth results. It is evident that other factors are needed. There is considerable work in the literature showing that certain concentrates will prevent the deficiency; however, the degree of improved growth reported by the different laboratories varies greatly.

Lepkovsky, Jukes, and Krause (5) were probably the first to show the need in rats of a factor distinct from thiamine, riboflavin, and vitamin B₆. Since this factor remained in the filtrate after the removal of riboflavin and vitamin B₆ by adsorption, it was called the "filtrate factor." Elvehjem and Koehn (6) had shown that the filtrate fraction was active in the prevention of chick dermatitis, and Lepkovsky *et al.* (5) found that concentrates which were free from vitamin B₆ (Factor 1) were active both for the prevention of dermatitis in chicks and growth in rats. For some

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time this fraction was related to the antipellagra factor but Jukes (7) threw some doubt on the identity of the chick factor and the antipellagra factor from studies on distribution. It is now known that nicotinic acid, the antipellagra factor, is distinct from the chick factor (Fouts *et al.* (8), Dann and Subbarow (9), Mickelsen, Waisman, and Elvehjem (10)).

The recognition of nicotinic acid as an essential factor immediately raised the question of whether it was the factor in the filtrate required by the rat. Several workers (9, 11-13) have shown that nicotinic acid cannot be used in place of the filtrate fraction for rats. Frost and Elvehjem (14) obtained some growth in rats with nicotinic acid, but their basal ration contained 12 per cent of corn. Von Euler *et al.* (15) got a response with cozymase and alkali-inactivated cozymase only after adding a yeast-fullers' earth filtrate preparation.

Most workers have removed vitamin B₆ by adsorption on fullers' earth from which it can be eluted with various reagents. It has been assumed that the filtrate and eluate each carried only one factor. Elvehjem, Koehn, and Oleson (16) found, however, that, when liver extract was used as starting material and treated with alcohol and ether to precipitate a large bulk of the soluble material, the filtrate produced no growth in rats receiving riboflavin and vitamin B₆ until the precipitate was also added. The filtrate contained the chick antidermatitis factor and nicotinic acid, but whether it contained another factor essential for rats in addition to that in the precipitate was not established. Further studies on the precipitate have been made by Frost and Elvehjem (14), and the fraction has been designated Factor W. The properties of the so called filtrate factor have been studied by Lepkovsky, Jukes, and Krause (5), Halliday and Evans (17), Edgar and Macrae (18), and Schultz and Mattill (19). It is evident that some of the properties reported are similar to those given for Factor W.

Further evidence for the multiple nature of the eluate fraction has been given by György *et al.* (20), who obtained a new deficiency in rats when they were fed purified concentrates of vitamin B₆ but not when crude concentrates were used. Other factors recently reported which are concerned with rat growth are the grass juice factor of Kohler, Elvehjem, and Hart (21) and vitamin

B₁ (Kline, Elvehjem, and Hart (22)). Thus it is evident that the water-soluble group consists of a larger number of individual essential factors than is usually recognized.

The results reported here have been obtained during the past 2 years from studies on several phases of the vitamin B complex but are combined in one paper because liver extract has been used in the preparation of all the concentrates. It may be valuable to compare our results with those obtained by other workers using concentrates from yeast and rice polishings.

Since rather crude concentrates have been used, it is possible that several factors are concerned. In fact the evidence points to this conclusion. Some time will be required before a complete picture is obtained, but a careful comparison of the work from different laboratories should decrease the time necessary to clarify the point.

EXPERIMENTAL

Rats from the stock colony were used in all of this work. The litters, with their mothers, were placed on raised screens during the last week of the suckling period. During this time the young were given access to the experimental ration only and the mothers removed to another cage for feeding. This enabled us to produce rats with a more uniform store of some of the factors concerned. At weaning the young were given the basal ration *ad libitum* or the basal ration plus the various supplements. All of the rats were kept in individual cages.

A number of different rations have been used but only a few will be described here. All the rations contained sucrose 76 to 78 gm., casein 18 gm., and Salts I, 4 gm. The supplements added in four of the rations are given in Table I.

The casein was prepared from skim milk, reprecipitated three times, and extracted four times with boiling alcohol. Salts I has been described by Phillips and Hart (23). Several different preparations of thiamine and riboflavin were used throughout the work, but in the more recent series Merck's crystalline vitamin B₁¹ and synthetic riboflavin (Hoffmann-La Roche) have been used. Crystalline riboflavin, prepared from milk concentrates and kindly

¹ We are indebted to Dr. R. T. Major, Merck and Company, Inc., for a generous supply of vitamin B₁.

furnished by Dr. S. Lepkovsky, was used in many of the earlier experiments. The carotene was fed as a water suspension at a level twice that recommended by Guilbert *et al.* (24). The percomorph oil supplied about 6000 i. u. of vitamin A per rat per week. Eastman's nicotinic acid and choline were used. The corn fatty acids were prepared by saponification and acidification of Mazola oil, followed by several washings and reprecipitation.

Rats placed on either of the above rations grew very poorly. Chart I shows typical results obtained with rats on the various rations. The growth curve for only one rat on each ration is given

TABLE I
Supplements Added to Basal Components of Rations

Supplements	Ration			
	J23	J24	J29	J30
	Per 100 parts of ration			Per rat per day
Thiamine.	0.12 mg.	0.12 mg.	0.12 mg.	5 micrograms
Riboflavin.	0.12 "	0.12 "	0.12 "	20 "
Nicotinic acid.			300 "	1 mg.
Choline.			500 "	1 "
Corn fatty acids.		2 gm.		
	Per rat per wk.			
Carotene.	35 micrograms			
Irradiation.	15 min.			
Percomorph oil.		2 drops	2 drops	2 drops

but identical results have been obtained with large numbers of rats. When these rations were supplemented with liver extract, good growth was generally obtained. The growth obtained when Rations J23 and J24 were supplemented with 0.25 gm. of liver extract per day or 2 per cent in the ration is shown in Chart I for comparison. It is evident that liver extract supplies most of the missing factors, although restricted growth has been obtained under certain conditions. These results will be discussed later.

Typical acrodynia developed in over 90 per cent of all the rats placed on either of these rations in 4 to 6 weeks. The region about the nose became erythematous, denuded, and eroded. The nose was usually moist owing to a nasal discharge. The paws became

red, swollen, and raw. Only a few cases of encrusted ears were observed. These results agree with those obtained by practically all workers using similar diets for the production of vitamin B₆ deficiency. Although these diets are low in several factors, the dermatitis is the first symptom to appear and can therefore be used with some reservation for vitamin B₆ assays.

When the rats were allowed to continue on the basal rations, some rats died but all those that survived showed additional symp-

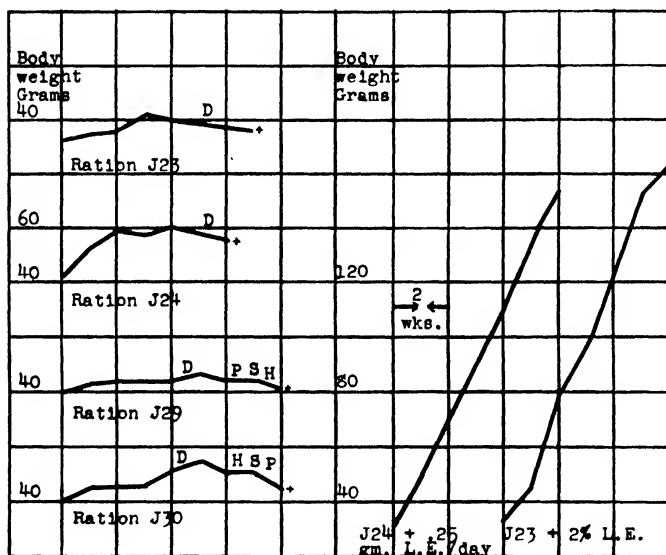


CHART I. The growth and symptoms obtained in animals receiving the basal rations alone and supplemented with liver extract. D, indicates the occurrence of acrodynia; S, spectacled eye; P, paralysis; H, hemorrhagic disease.

toms. An erosion set in about the eyes, imparting to the rats a spectacled appearance. The lids became denuded and scaly. In many cases the eyes were closed by a sticky exudate. A second condition was characterized by subcutaneous hemorrhages or purpura of the paws and bleeding from the nose. The animals did not survive long after these symptoms developed. Some of the animals showed a definite reduction in the hemoglobin content of the blood.

A large number of the rats also exhibited a very characteristic form of paralysis. The rats walked with a very high spastic gait as though there was a hypertonicity of the extensor muscles. They seemed to experience extreme difficulty in locomotion with some loss of equilibrium, due probably to the awkward position which they assumed.

The spectacled eye condition and paralytic condition are shown in Figs. 1 and 2. The sequence of the onset of the last three symptoms varied in different groups of rats, but they all became evident within a rather short period of time. A typical example of



FIG. 1



FIG. 2

FIG. 1. Typical spectacled eye condition. The rat was given Ration J29 until aerodynia developed and then the eluate was added, after which the dermatitis was cured and the eye condition appeared (Rat 10244).

FIG. 2. Typical hunched position of a rat suffering from the paralysis symptoms. The rat has severe dermatitis as well (Rat 11643).

the variation in order of development is shown in the rats on Rations J29 and J30 (Chart I). It is also interesting to note that the rats receiving nicotinic acid and choline generally survived longer after developing aerodynia and therefore were more likely to show the other symptoms.

The "spectacled eye" condition has been described by several investigators as a part of the so called "rat pellagra syndrome." Goldberger and Lillie (25) and Bourquin and Sherman (26) reported its occurrence on their rations. Robinson and Newton (27) produced this condition quite consistently. Lepkovsky, Jukes, and Krause (5) report a spectacled appearance in their rats on rations low in the "filtrate factor," thus narrowing down

the etiology of this condition. The rats of Sjollesma (28) and Karrer *et al.* (29) had an erosion about the eyes, which was cured with lactalbumin and flavin phosphate respectively. We have found this condition to occur along with or independently of acrodynia. This condition appears to be somewhat similar to that found in chicks suffering from dermatitis (30-32).

The hemorrhagic type of deficiency resembles the panmyelophthisis described by György *et al.* (20). György (33) later reported its prevention with nicotinic acid. We have found that nicotinic acid did not prevent the condition observed in our rats. The deficiency occurs on our basal ration alone or plus various supplements, although the incidence is much less than that of the other deficiencies. We cannot state at this time whether the condition is panmyelophthisis or not since the blood picture of the deficient animals has not been studied.

The paralysis observed is quite probably the same as that observed in vitamin B₄ deficiency (22). The work reported in this paper indicates that each of these deficiencies is due to the lack of separate essential factors.

Lepkovsky *et al.* (5) succeeded in separating vitamin B₆ from the "filtrate factor" by adsorbing the former on fullers' earth. We have utilized this procedure in our experiments. Our source material was a liver extract preparation from The Wilson Laboratories which was a by-product in the manufacture of the pernicious anemia fraction.² When fed to rats at a level of 2 per cent in the ration or 250 mg. per day, a fair rate of growth was obtained and no deficiency symptoms appeared. The procedure adopted for the fractionation of the liver extract was as follows: To 100 gm. of liver extract were added 20 cc. of water, 1 liter of acetone, and 1 cc. of concentrated H₂SO₄. The mixture was shaken in a 2 liter bottle vigorously for 10 minutes according to the method of Frost and Elvehjem (14). The solids were allowed to settle and the supernatant liquid was decanted off. This process was repeated six times. The combined extracts were concentrated *in vacuo* and the H₂SO₄ was removed with Ba(OH)₂. The solution was filtered, diluted to 800 cc. with water, acidified with 20 cc. of concentrated H₂SO₄, and shaken with 50 gm. of English fullers'

² The liver extract preparation was supplied to us by Dr. David Klein. The Wilson Laboratories, Chicago.

earth. The adsorbate was filtered off and washed with water. The wash water was added to the filtrate. The process was repeated with three additional batches of fullers' earth. The combined filtrate and washings were neutralized with $\text{Ba}(\text{OH})_2$, filtered, and concentrated *in vacuo*.

The combined adsorbates were washed once with a liter of acidified water. The elution was carried out by shaking the ful-

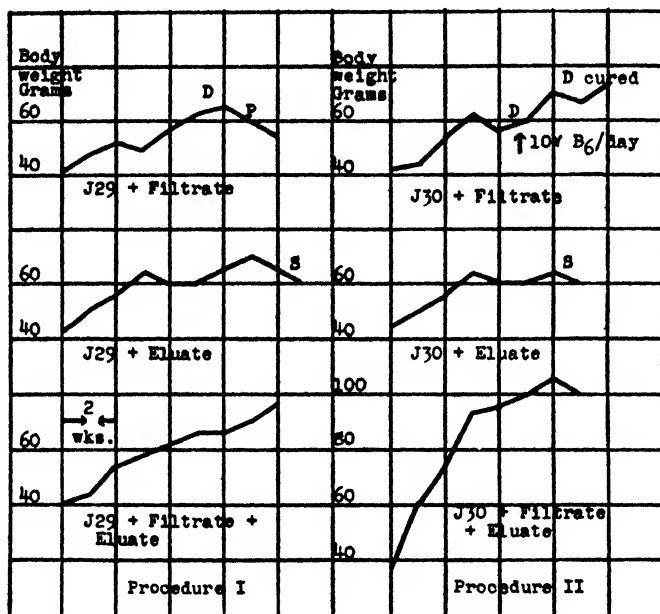


CHART II. The effect of the liver fractions on the growth of animals and the symptoms obtained. Procedure I, fullers' earth eluted with $\text{Ba}(\text{OH})_2$; Procedure II, fullers' earth eluted with pyridine. Symbols as in Chart I.

lers' earth twice with 500 cc. of 1 N $\text{Ba}(\text{OH})_2$ for $\frac{1}{2}$ hour each time. The fullers' earth was filtered off, washed, and discarded. The combined eluates were neutralized with H_2SO_4 , filtered, and concentrated *in vacuo*. These preparations will be referred to as the "filtrate" and "eluate" respectively.

The results obtained when these concentrates were added to Ration J29 are shown in Chart II. When the filtrate alone was

fed, the rats developed typical acrodynia in 5 to 6 weeks. Their growth was not promoted by this supplement. However, the spectacled eye condition did not develop. When the eluate alone was fed, the acrodynia was prevented but the erosion about the eyes did appear. The feeding of both fractions prevented the appearance of any visible symptoms but the combined fractions did not promote normal growth in the animals, indicating that some factor was destroyed by the procedure or was not eluted from the fullers' earth. In this way the factors preventing acrodynia and the eye erosion could be separated into two fractions.

Since there were several possibilities for loss of additional growth factors in the above separation, a less drastic procedure was used. 100 gm. of liver extract were diluted to 800 cc. with water and placed in a 2 liter flask. 40 gm. of English fullers' earth were added and the contents shaken by a gentle rotatory motion for $\frac{1}{2}$ hour. The fullers' earth was filtered, washed, and the wash water combined with the filtrate. This process was repeated two more times. The filtrate was concentrated *in vacuo* and made to definite volume. The fullers' earth was eluted with pyridine and methyl alcohol in the usual way.

Typical results obtained with these fractions when added to Ration J30 are shown in Chart II. No better growth was obtained on the individual fractions, but when they were fed in combination a greater initial growth was observed. However, the rats again ceased growing after the first few weeks. The symptoms of acrodynia and spectacled eye were prevented completely when both the filtrate and eluate were fed but evidently even this mild treatment of the liver extract allowed the partial loss of other essential factors.

It is very probable that the growth-promoting property of the liver extract which was lost during fractionation is related to Factor W. Frost and Elvehjem (14) found considerable loss of this factor when certain types of adsorbing agents were used. The addition of Factor W concentrates actually produced definite improvement in the rate of growth. For a time we hoped to introduce purified concentrates of this factor into the basal ration in order to intensify some of the other symptoms. Growth responses were obtained with both the mercury precipitate and

mercury filtrate fractions of Frost and Elvehjem but symptoms of acrodynia were also prevented. Halliday and Evans (34) found that the alcohol-ether precipitate from whole liver contained large amounts of vitamin B₆, and evidently some B₆ is carried along in the final concentration procedures. The complete lack of growth which resulted in these studies with concentrates of vitamin B₆ sufficiently potent to prevent acrodynia gives further evidence for the existence of Factor W distinct from vitamin B₆. Pure preparations of Factor W will be valuable additions to the basal ration.

Further evidence for the existence of lesions other than those of acrodynia were obtained through the use of crystalline vitamin B₆. A generous supply of the crystalline material was kindly furnished by Dr. Lepkovsky. In confirmation of Lepkovsky's report, the crystals were found highly active at a level of 10 micrograms per day. A comparison of the results obtained with the eluate and the crystals on Ration J30 alone is given in Chart III. When either supplement was used, the spectacled eye condition appeared after the acrodynia was cured. This has been true in almost all of our rats. The response obtained by adding the vitamin B₆ crystals to Ration J30 plus filtrate is also shown in Chart III. In this case a more definite growth response resulted, but it does not compare with that reported by Lepkovsky (1). The growth factor is evidently more labile in the liver extract which we used than in the rice bran extract or liver preparation used in Lepkovsky's work. When vitamin B₆ crystals were added to Ration J30 from the beginning, all the additional symptoms except acrodynia appeared. Other typical results are shown in Chart III. In a total of twenty rats on this regimen, the incidence of the hemorrhagic condition has not been so high as the spectacled eye condition. However, since the hemorrhagic condition appeared more frequently when the crystals were used than when the concentrate was fed, it appears that the factor concerned must be adsorbed on the fullers' earth along with vitamin B₆. This result is in agreement with that reported by György. The possibility that the hemorrhagic condition is related to vitamin K deficiency has not been definitely eliminated.

It is evident from the above work that the filtrate fraction definitely carries the factor necessary for the prevention of the

spectacled eye condition and may or may not carry appreciable amounts of other growth factors. Since Koehn and Elvehjem showed the fraction of the crude alcohol-ether precipitate (Factor W) to be completely inactive in the prevention of chick dermatitis, it appears that the spectacled eye factor is most likely to be the factor in the filtrate which might be related to the chick anti-dermatitis factor.

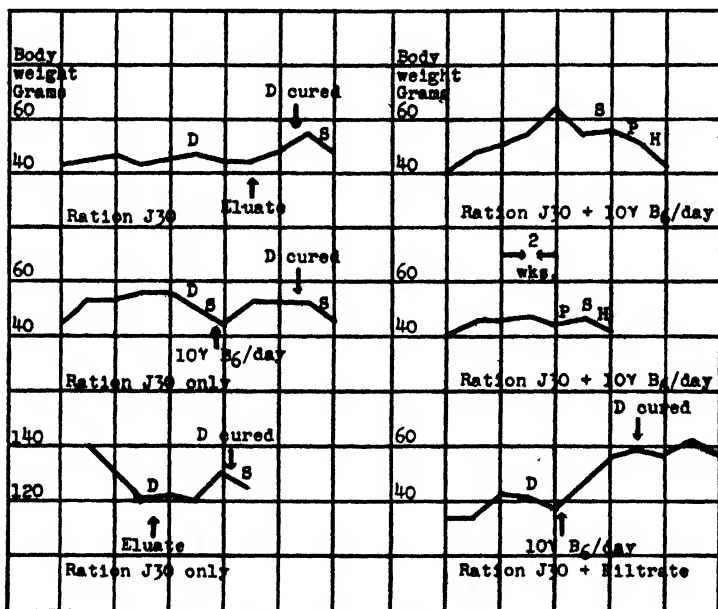


CHART III. The occurrence of deficiency symptoms in rats receiving various supplements. Symbols as in Chart I.

Crude concentrates prepared from liver extract and shown to be highly active in the prevention of chick dermatitis (Woolley, Waisman, Mickelsen, and Elvehjem (35)) brought about rapid cure of the spectacled eye condition but very little improvement in growth. More highly purified fractions did not give as consistent results. Definite conclusions regarding the identity of the factor necessary for the prevention of the spectacled eye condition in rats and the chick antidermatitis factor must be withheld until more highly purified fractions are used.

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In order to remove the suspicion that the eye condition might be related to a faulty vitamin A ingestion, the livers from some of the rats showing typical symptoms were removed and analyzed for vitamin A by the SbCl_3 method as outlined by Carr and Price (36). The following results were obtained: for Rats 10714, 11811, 11814, and 11834, 275, 148, 150, and 98 total blue units respectively.

The inclusion of our filtrate or crystalline vitamin B_6 in the rations will not prevent the paralysis. The factor preventing the paralysis is present in our eluate. One rat was cured rapidly when fed 0.6 gm. per day of dehydrated barley-grass. McHenry (37) reported that rats deficient in choline exhibited a paralysis similar in appearance to that attributed to a deficiency of vitamin B_4 . However, we have seen many cases of the paralysis in rats receiving 1 mg. of choline per day. Work from this laboratory indicates that choline is inactive in preventing vitamin B_4 deficiency in chicks.^a

Sjollema (28) reported that leucine, isoleucine, and lactalbumin would cure rat acrodynia. We tested these substances on rats receiving Ration J29 or Ration J29 plus the filtrate fraction and observed the effects on all the symptoms described above. The amino acids were fed at levels of 50 and 100 mg. per day to each rat and the lactalbumin was fed at 4 and 8 per cent of the ration. Harris' vitamin-free lactalbumin and synthetic amino acids were used in these experiments. The results obtained with some of the rats are shown in Table II. In most cases the above supplements were entirely inactive in the prevention or cure of acrodynia, but in a few cases a slight to definite activity was noted, but in all of these cases the effect was only temporary. The results were complicated by the appearance of other deficiency symptoms which may have some effect on the results obtained. Lunde and Kringstad (38) have been unable to confirm Sjollema's results.

The poor growth obtained with the fractions from the liver extract needs further consideration in light of the results obtained by other workers. Edgar and Macrae (39) report the same rate of growth when they used a yeast extract autoclaved at pH 5.0 as when they used the unautoclaved material. We have repeated

^a Bird, Oleson, Elvehjem, and Hart, unpublished data.

this treatment with liver extract and obtained quite different results. Growth records of rats fed Ration J29 plus 2 per cent untreated liver extract and the same material autoclaved for 5

TABLE II

Effect of Leucine, Isoleucine, and Lactalbumin Additions on Acrodynia and Other Deficiency Symptoms; Basal Ration J29 Used Throughout

Rat No.	Filtrate	Supplement	Acrodynia	Other symptoms
Additions made from beginning				
11321	—	125 mg. leucine per day	Not prevented	Paralysis
11322	—	12% casein	“ “	“ hemor- rhagic condition
11323	—	8% lactalbumin	“ “	Paralysis
11324	—	125 mg. isoleucine per day	Slight	
10963	—	4% lactalbumin	Not prevented	Paralysis
10883	+	25 mg. leucine + 25 mg. isoleucine per day	No dermatitis	Hemorrhagic con- dition
10881	—	50 mg. leucine per day	Not prevented	
10873	+	4% lactalbumin	“ “	Paralysis
Additions made after dermatitis developed				
10711	+	50 mg. leucine per day	Slight improvement	
10291	—	1% isoleucine	Marked “	Spectacled eye
10292	—	0.5% “	No improvement	
10664	+	4% lactalbumin	Some improvement	Hemorrhagic con- dition
10871	+	50 mg. isoleucine per day, increased to 100 mg.	Slight “	Paralysis
10874	+	50 mg. leucine per day, increased to 100 mg.	“ symptoms; nearly cured but symptoms reappeared	Paralysis; specta- cled eye

hours at 120° and 15 pounds pressure are shown in Chart IV. It is evident that a very large percentage of the growth-promoting property of the liver extract is destroyed by autoclaving. The

irradiation of the liver extract with a quartz mercury vapor lamp also diminished the activity of the liver extract to some extent. However, in each case the rats showed no symptoms except the retarded growth.

It is quite likely that the stability of certain factors varies in different products, but we must not overlook the fact that the rations used in the different laboratories have varied considerably

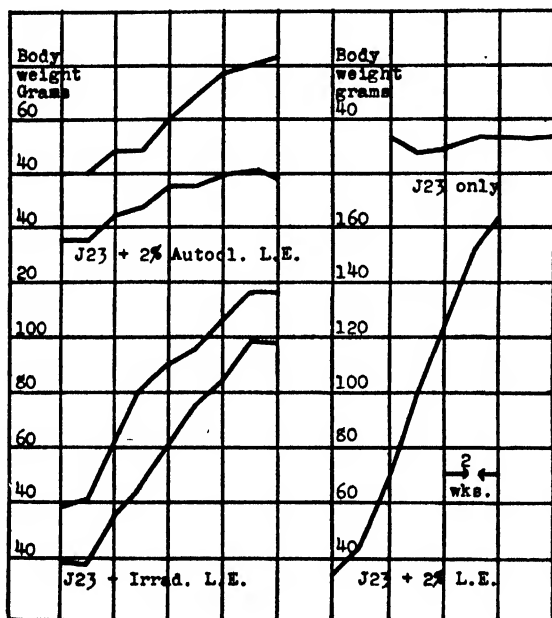


CHART IV. The growth-promoting power of liver extract when autoclaved and irradiated.

in composition. Lepkovsky (1) used 10 parts of lard and Edgar and Macrae (39) used 12 parts of cottonseed oil and 3 parts of lard. It is known that the kind and amount of fat in the ration has a definite effect on the incidence and cure of vitamin B₆ deficiency in rats (40-43). Birch (44) has recently discussed the relation of fat and vitamin B₆.

We too have found that the addition of 0.25 cc. of corn oil or lard to Ration J30 per rat per day causes definite improvement in the

acrodynia, but little if any growth results. The incidence of spectacled eye, paralysis, and hemorrhagic condition was also greatly reduced in the rats getting the fat addition. In addition to this effect, certain fats seem to give improved growth even in addition to liver extract. Results in Chart I show that 0.25 gm. of liver extract per rat per day in addition to Ration J23 and J24 produced a growth of about 3 gm. per day. Similar results have been obtained when Rations J29 and J30 were used for rats from certain litters. Other rats, however, have grown poorly on these

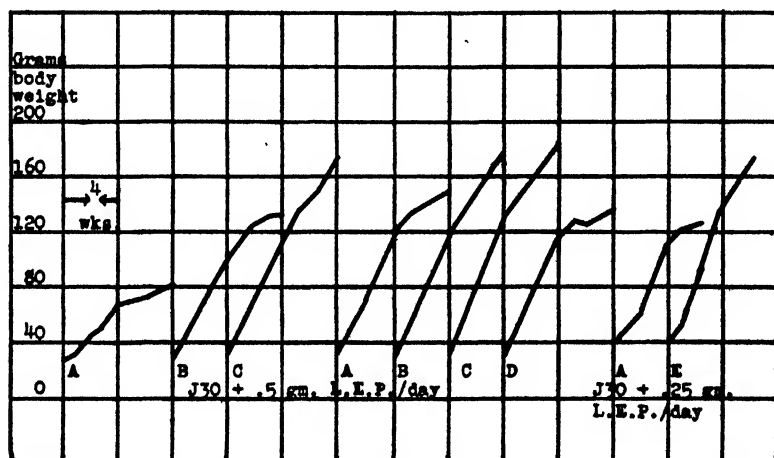


CHART V. The effect of adding fat and dextrin to the basal diets supplemented with liver extract. A indicates no supplement; B, 0.12 gm. of linoleic acid per day; C, 0.25 gm. of corn oil per day; D, 0.25 gm. of trilaurin per day; E, sucrose replaced by extracted dextrin.

rations plus liver extract at 0.25 gm. per day or even 0.5 gm. per day.

Some of the typical results obtained when fats were added in addition to liver extract are shown in Chart V. Rats from two litters are shown to illustrate the difference in response to liver extract alone. The addition of 0.25 cc. of corn oil per rat per day has always given a definite improvement in growth regardless of the response on the control ration. Since almost 50 per cent of the fatty acids in corn oil may be linoleic acid, it was fed to some of the rats at a level of 0.12 cc. per day. The linoleic acid also produced

an increased rate of growth, but the response to it has never been as complete or as uniform as with corn oil. In contrast to the effect of corn oil and linoleic acid, trilaurin has given completely negative results. The effects which have been observed may be related directly to a deficiency of unsaturated fatty acids, since both our ration and liver extract are undoubtedly deficient in unsaturated fatty acids. However, we have dealt only with growth effects and have not seen any of the typical symptoms generally encountered in unsaturated fatty acid deficiency.

Another interesting observation in this connection is that rats which show retarded growth on Ration J29 plus liver extract resume normal growth when the sucrose in the ration is replaced by an equal amount of dextrin. Rats placed on the dextrin diet from the start have always grown better than those placed on the sucrose diet. At first we felt that the dextrin might be supplying some fat-soluble material but when the dextrin was extracted with boiling alcohol for 2 hours and boiling ether for 2 hours the growth stimulation was not lost; in fact the rats receiving the extracted dextrin grew better than those getting the crude material. Again we are at a loss to explain the stimulating effect. It is, however, entirely possible that the presence of dextrin may influence the intestinal flora and allow synthesis of the lacking essential factors. Taylor and Lehrman (45) found starch to contain appreciable amounts of unsaturated fatty acids.

DISCUSSION

The use of chicks and dogs in addition to rats as experimental animals in studies on the fractionation of the members of the vitamin B complex has aided progress in this field to a considerable extent. It is logical to expect that different symptoms will be observed as the first deficiency when different animals are used and when slightly modified basal rations are fed. Thus we find that on rations relatively low in the vitamin B complex plus thiamine and riboflavin the first noticeable symptom in rats is acrodynia, in dogs black tongue, and in chicks chick dermatitis. Each deficiency is due to a separate and distinct factor. Temporary difficulties have arisen from conclusions that crude concentrates active in the treatment of deficiencies in two different species contain but one factor. There has been considerable hesitancy in

accepting new and distinct factors, but we now have many examples where one concentrate carried several different factors. One of the best examples was the close correlation between the chick antidermatitis activity and anti-black tongue activity of concentrates from liver extract until the final isolation of nicotinamide. Differentiation of specific factors in various vitamin B concentrates has been discussed very recently by Dann and Subbarow (9) and Fouts, Helmer, Lepkovsky, and Jukes (46). The work reported in this paper is merely an extension of such results.

Evidence is presented to show the "eluates," "filtrates," and "precipitates" prepared by different workers may be complex mixtures of active substances. The eluate fraction as prepared in this study contains not only vitamin B₆ but the factors necessary for the prevention of paralysis and the hemorrhagic condition. The use of pure vitamin B₆ allows the development of the additional symptoms. It carries very little if any of the growth factor, which we have designated Factor W.

The filtrate fraction carries the factor necessary for the prevention of the spectacled eye condition but a reduced amount of Factor W and possibly other growth factors. The filtrate fractions prepared by Lepkovsky and Jukes (32) and Edgar and Macrae (18) seem to carry the growth-promoting factors. It is obvious that the term filtrate factor cannot be used unless it is used for only one of these factors.

The filtrate fraction also may carry the chick antidermatitis factor and nicotinic acid. That is why it is impossible at the present time to decide whether the chick antidermatitis factor and rat spectacled eye factor are separate factors and whether nicotinic acid is essential for the rat. If the essential nature of nicotinic acid is to be tested, all the other factors must be supplied and it is not possible yet to prepare all the factors free of nicotinic acid.

Similarly the alcohol-ether precipitate of Elvehjem, Koehn, and Oleson (16) and even more purified preparations of Factor W carry vitamin B₆ and probably other factors. In the work on Factor W the relation of vitamin B₆ is eliminated because the basal ration carries adequate amounts of this vitamin. The differentiation of factors related to specific pathological syndromes is more readily attained than separation of those related only to

growth. Therefore the growth-promoting property of liver extract may again be multiple in nature. Further work on Factor W in this laboratory indicates that this may be the case.

The work is further complicated by the importance of fat and dextrin for optimum growth when liver extract is used as the source of the water-soluble vitamins. Whether the growth effect is due to a separate entity or whether the effect is related to unsaturated fatty acids and vitamin B₆ cannot be decided until more work is done. Each of the factors included in this paper needs a great deal more study but we have intentionally discussed all the factors in one paper in order to point out possible relationship.

SUMMARY

1. Four different deficiency symptoms have been produced in rats on synthetic diets low in the vitamin B complex but adequate in thiamine, riboflavin, choline, and nicotinic acid. They are acrodynia, paralysis, hemorrhagic disease, and spectacled eyes.

2. Crystalline vitamin B₆ was very active in preventing acrodynia, but inactive in preventing paralysis, hemorrhagic disease, or spectacled eye condition.

3. The relation of these deficiencies to known members of the vitamin B complex is discussed.

4. Further evidence of the need of Factor W for growth was produced.

5. The importance of the dietary fat and carbohydrate in relation to growth was demonstrated.

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THE SYNTHESIS OF THE NEXT HIGHER AND LOWER HOMOLOGUES OF *l*-CARNOSINE: γ -AMINOBTUTYRYL- *l*-HISTIDINE AND GLYCYL-*l*-HISTIDINE

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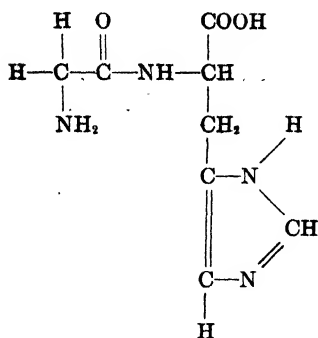
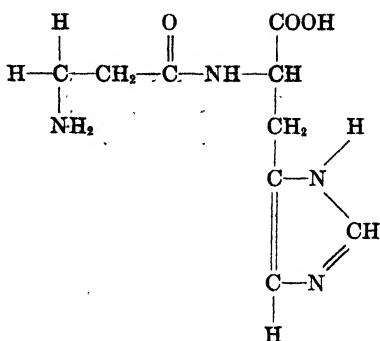
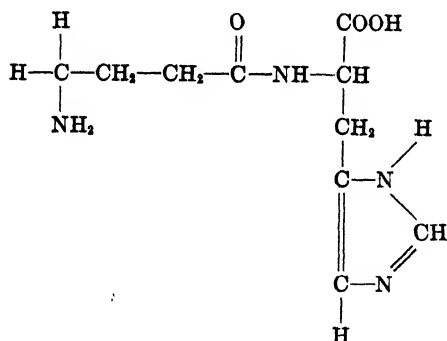
The demonstration that α -*l*(+)-alanyl-*l*(-)-histidine and α -*d*(-)-alanyl-*l*(-)-histidine did not exert an effect on the blood pressure in contrast to carnosine, β -alanyl-*l*(-)-histidine, emphasized the importance of the presence of the amino group in the β position of the β -alanyl moiety of carnosine (1). However, the question arose as to whether the pharmacodynamic action was dependent upon the presence of the β -amino group *per se* or whether it was really due to the fact that the β -alanine had an amino group in the terminal position. We wondered whether other peptides of histidine with the terminal amino groups in the acyl portion might likewise possess depressor activity. To throw some light on this question we decided to synthesize the next higher and lower homologues of *l*-carnosine, namely γ -amino-butyryl-*l*-histidine and glycyl-*l*-histidine, respectively, and compare their effect on the blood pressure with *l*-carnosine. The relation of these peptides to *l*-carnosine is shown in the accompanying structures.

A synthesis of glycyl-*l*-histidine has already been reported by Abderhalden and Geidel (2). Their peptide, prepared through amination of the chloroacetyl derivative of histidine, was an amorphous preparation and no crystalline derivatives were reported.

The synthesis of the crystalline glycyl-*l*-histidine hydrochloride reported in the present paper was accomplished by condensation of carbobenzoxyglycyl chloride with histidine methyl ester by the general procedure of Bergmann and Zervas (3).

Histidine Peptides

γ -Aminobutyryl-*l*-histidine was synthesized in like manner. It was isolated as the crystalline sulfate.

Glycyl-*l*-histidine β -Alanyl-*l*-histidine γ -Aminobutyryl-*l*-histidine

The carbobenzoxy- γ -aminobutyric acid employed in this synthesis was prepared from β -bromopropionic acid which was converted to the nitrile and then reduced to give γ -aminobutyric acid. The highly insoluble carbobenzoxy derivative was employed to isolate the amino acid from the reaction mixture.

Both peptides, in 20 times the dose of *l*-carnosine, were found to possess no effect on the blood pressure of cats under amytal anesthesia. The results bring out clearly the importance of the amino group in the β position of the acyl moiety of carnosine. Apparently the presence of the amino group in the terminal position is not the crucial factor. These results along with those pre-

viously reported for the α -alanyl peptides of *L*-histidine (1) and for *D*-carnosine (4) demonstrate an amazing specificity of the structure of *L*-carnosine in relation to its depressor action.

EXPERIMENTAL

Preparation of Carbobenzoxyglycyl-L-Histidine—16 gm. of histidine methyl ester dihydrochloride were converted to the free methyl ester by the method of Fischer and Cone (5). The oily methyl ester was dissolved in 150 cc. of absolute chloroform and was kept at 0° during the preparation of the carbobenzoxyglycyl chloride.

7 gm. of the carbobenzoxyglycyl chloride prepared according to Bergmann and Zervas (3) were dissolved in 50 cc. of ice-cold absolute chloroform. The solution was added to the histidine methyl ester solution. An immediate reaction took place with the separation of oily histidine methyl ester monohydrochloride which soon crystallized and could be filtered. 6.6 gm. of histidine methyl ester monohydrochloride were obtained, which represents about half of the histidine methyl ester used. The filtrate from the histidine methyl ester monohydrochloride was concentrated *in vacuo* to remove the chloroform. The residue was dissolved in 150 cc. of dioxane and was reconcentrated *in vacuo* until about 50 cc. of distillate had collected. 50 cc. of H₂O and 9 cc. of 4 N NaOH were added to the residual solution. After the solution had been allowed to stand for $\frac{1}{2}$ hour at room temperature, 9 cc. of 4 N H₂SO₄ were added. This solution was concentrated to dryness *in vacuo*, and the residue was extracted with 300 cc. of boiling absolute alcohol in three portions. The combined alcoholic extracts were concentrated *in vacuo* to 100 cc. and the resulting solution was cooled 24 hours. The crystalline carbobenzoxyglycyl-*L*-histidine obtained was filtered and was washed with several small portions of cold absolute alcohol. The yield was 8 gm., which represents 70 per cent of the theoretical yield, allowance being made for the histidine recovered. The compound melted at 175° (corrected) and possessed a specific rotation of $[\alpha]_D^{25} = +22^\circ$ for a 1.5 per cent aqueous solution. For analysis the compound was recrystallized once from absolute alcohol and dried *in vacuo* at 70°.

C₁₈H₁₈N₄O₄. Calculated, N 16.18; found, N 15.96

Preparation of Glycyl-L-Histidine Hydrochloride—6 gm. of carbobenzoxyglycyl-L-histidine were dissolved in 20 cc. of water and 2 equivalents of HCl were added. The reduction of carbobenzoxyglycyl-L-histidine hydrochloride by hydrogen in the presence of palladium black was carried out by bubbling hydrogen through the solution until no more CO_2 was evolved. The catalyst was removed by filtration and the solution was concentrated to dryness *in vacuo*. The residue was dissolved in the minimum amount of water and 5 cc. of aniline were added. Upon the addition of 5 volumes of absolute alcohol, crystallization of the monohydrochloride of glycyl-L-histidine took place. The yield was 3.9 gm., which represents 85 per cent of the theoretical yield. The compound melted at 175° (corrected) and had a specific rotation of $[\alpha]_D^{26} = +25^\circ$ for a 1 per cent aqueous solution. The material dried in air contained 1 molecule of water of crystallization.

$\text{C}_8\text{H}_{12}\text{N}_4\text{O}_5 \cdot \text{HCl} \cdot \text{H}_2\text{O}$	Calculated.	N 21.01, Cl 13.30
	Found.	" 21.20, " 13.38

Preparation of Carbobenzoxy- γ -Aminobutyric Acid—25 gm. of β -bromopropionic acid were dissolved in 100 cc. of water to which had been added 1 equivalent of NaOH. 8.5 gm. of NaCN were then added to the solution. The mixture was heated at 40 – 50° for 1 hour and then reduced with hydrogen in the presence of Raney's nickel catalyst. After the catalyst had been removed by filtration, the solution was placed in a 500 cc. flask which was equipped with a stirrer and surrounded by an ice bath. 40 gm. of carbobenzoxy chloride were added in four portions at 5 minute intervals. During the addition of the carbobenzoxy chloride the reaction mixture was vigorously stirred and 6 N NaOH was added from time to time to keep the solution alkaline to phenolphthalein. The stirring was continued for 30 minutes after the addition of carbobenzoxy chloride was complete. The solution was then extracted with ether to remove certain impurities and the aqueous layer was acidified with HCl. Upon acidification, a heavy oil separated which was removed from the aqueous portion by extraction with ether. The ether solution was reextracted with Na_2CO_3 solution and the aqueous layer again acidified. The oil which separated was extracted successively with two 50 cc. portions of chloroform. The chloroform solution was dried over Na_2SO_4 .

Addition of 2 volumes of petroleum ether to the anhydrous chloroform solution caused the separation of an oil which soon crystallized. The yield was 20 gm., which is 50 per cent of the theoretical amount. The crude compound melted at 64° (corrected). For analysis it was recrystallized from chloroform by the addition of petroleum ether. The purified product melted at 66° (corrected).

$C_{13}H_{15}NO_4$. Calculated, N 5.90; found, N 5.92

Preparation of γ -Aminobutyryl-L-Histidine Sulfate—16 gm. of histidine methyl ester dihydrochloride were converted to the free ester by the method of Fischer and Cone (5). The free ester was dissolved in 150 cc. of absolute chloroform and kept at 0°.

A mixture of 12 gm. of carbobenzoxy- γ -aminobutyric acid, 40 cc. of dry ether, and 12 gm. of PCl_5 was shaken for 15 minutes at 0°. The mixture was filtered and the filtrate was concentrated *in vacuo* until about two-thirds of the ether had been removed. 100 cc. of petroleum ether, which had previously been cooled to -20°, were added to the concentrated solution. The oily layer of acid chloride which separated was washed twice with 100 cc. portions of cold petroleum ether. The acid chloride was then immediately dissolved in 25 cc. of cold chloroform and added to the previously prepared histidine methyl ester solution. The mixture was shaken for several minutes and was then extracted with water. The chloroform layer was concentrated to a thick oil *in vacuo*. After the residue was dissolved in 50 cc. of dioxane and the last traces of chloroform had been removed *in vacuo*, 24 cc. of 4 N NaOH and 50 cc. of water were added. This mixture was shaken for 5 minutes and was then neutralized with 24 cc. of 4 N H_2SO_4 . The solution was concentrated to dryness *in vacuo* and the residue was thoroughly extracted with hot absolute alcohol. The residue was dissolved in 30 cc. of water and 15 cc. of 4 N H_2SO_4 . The solution was reduced in the manner described for the reduction of carbobenzoxyglycyl-L-histidine. The catalyst was removed by filtration and the filtrate was neutralized with $Ba(OH)_2$. After the $BaSO_4$ was filtered, the resulting solution was evaporated to dryness and the residue was dissolved in the minimum amount of water. Enough H_2SO_4 was added to make the solution just acid to Congo red. The addition of 4 volumes of alcohol brought about the crystallization of the sulfate of γ -aminobutyryl-L-his-

tidine. The compound was recrystallized twice from water by the addition of alcohol. 3.7 gm. of recrystallized compound which melted at 235° (corrected) were obtained. This represents 32 per cent of the theoretical yield based on half of the histidine methyl ester used. The other half of the histidine methyl ester used was recovered as the monohydrochloride. The peptide sulfate had a rotation of $[\alpha]_D^{25} = +5^\circ$ for a 1 per cent aqueous solution. For analysis the compound was dried *in vacuo* at 75°.

$C_{10}H_{18}N_4O_8 \cdot H_2SO_4$. Calculated. N 16.56, SO₄ 28.39
Found. " 16.37, " 28.29

SUMMARY

Glycyl-*l*-histidine and γ -aminobutyryl-*l*-histidine were synthesized by condensation of the acid chlorides of the carbobenzoxy derivatives of glycine and γ -aminobutyric acid respectively with histidine methyl ester. After saponification and reduction of the condensation product, the crystalline hydrochloride of the glycyl-*l*-histidine and the crystalline sulfate of γ -aminobutyryl-*l*-histidine were isolated.

Neither peptide in 20 times the dose of *l*-carnosine was capable of lowering the blood pressure of cats under amytal anesthesia.

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5. Fischer, E., and Cone, L. H., *Ann. Chem.*, **363**, 107 (1908).

PARTIALLY O-METHYLATED HEXITOLS

I. 1,2,3,5,6-O-PENTAMETHYL-*d*-SORBITOL

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(Received for publication, October 6, 1938)

Two methods are available for the study of the position of the union between the two components of an aldobiase (or of aldobionic acids). First, the oxidation of the aldehydic group with subsequent methylation and hydrolysis and second, the reduction of the aldehydic group with subsequent methylation and hydrolysis.

To date, the first method alone has found application. It is felt, however, that in many instances, if not in all, the reduction method might offer many advantages. This would be particularly the case if the partially methylated hexitols were available as reference substances. With this aim in view the synthesis of partially substituted hexitols is now initiated.

The most frequent place of union is carbon atom 4 of the component having the aldehydic group unsubstituted and by virtue of this the synthesis of 1,2,3,5,6-O-pentamethyl sorbitol seemed most desirable and incidentally is the easiest to accomplish.

The structure of cellobiose being definitely established as 4-*d*-glucosido-*d*-glucose, it served as the starting material for the preparation of β -4-*d*-glucosido-*d*-sorbitol. The non-crystalline derivative of it has been prepared by Karrer and Büchi.¹ The crystalline substance and its O-nonamethyl derivative have been prepared by Levene and Kuna.² The latter substance on hydrolysis yields 1,2,3,5,6-O-pentamethyl sorbitol and 2,3,4,6-O-tetramethyl-*d*-glucose.

The separation of these substances by fractional extraction or

¹ Karrer, P., and Büchi, J., *Helv. chim. acta*, **20**, 86 (1937).

² Levene, P. A., and Kuna, M., *Science*, **85**, 550 (1937).

by distillation was found impossible. However, when the product of hydrolysis is oxidized by the Willstätter and Schudel method, and the concentrated product of the oxidation is rendered alkaline, then the sorbitol derivative is readily extracted with chloroform.

The 1,2,3,5,6-O-pentamethyl-*D*-sorbitol is obtained as a viscous syrup which distils at 128–133°, $p = 3.0$ mm., $n_D^{25} = 1.4439$, and $[\alpha]_D^{25} = -10.1^\circ$ (in absolute ethanol).

EXPERIMENTAL

*Reduction of Cellobiose to β -4-Glucosidosorbitol*²—20 gm. of cellobiose were dissolved in 200 cc. of water and about 5 gm. of Raney's catalyst were added. This was placed in an all-glass cylinder with a small hole bored in the middle and then put into a high pressure hydrogenation apparatus made by the American Instrument Company, Inc. This was rocked at a speed of 29 times per minute for 16 hours at a temperature of 125° and hydrogen pressure of 3500 pounds per sq. inch (230 atmospheres). The catalyst was filtered and the filtrate was concentrated to a syrup under reduced pressure. The material was dissolved in methanol and then ethanol was added to opalescence. A few cc. of water were then added³ and, on cooling, crystals separated. These were filtered and dried overnight in a desiccator. Yield 18 gm. M.p. 133°.

$$[\alpha]_D^{25} = \frac{-0.99^\circ \times 100}{1 \times 11.4} = -8.68^\circ \text{ (in water)}$$

5.021 mg. substance: 7.699 mg. CO₂ and 3.240 mg. H₂O
 C₁₂H₂₄O₁₁. Calculated. C 41.83, H 7.03
 344.2 Found. " 41.81, " 7.21

Nonamethyl β -4-Glucosidosorbitol—25 gm. of β -4-glucosidosorbitol were methylated according to West and Holden⁴ with some modifications. Ground glass joints were used throughout, and a 1 liter 3-neck flask was used for the reaction. The stirrer was made of stainless steel and was supported by four ball bearings. The blades of the stirrer were 30 mm. long and 15 mm. wide, with a round notch cut in the top of one blade and the bottom of the other. The stirrer was driven by a 100 watt Hamilton Beach motor with a top speed rating of 12,000 R.P.M. without a load.

³ Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **125**, 355 (1938).

⁴ West, E. S., and Holden, R. F., *J. Am. Chem. Soc.*, **56**, 930 (1934).

The reaction was carried on with all of the resistance cut out. A drawing of the apparatus is given in Fig. 1. The stop-cocks of the dropping funnels were attached to a lock screw device by which the rate of flow was kept constant, in spite of the vibration.

The chloroform extract was evaporated to a syrup and dried in a high vacuum. The material in this state was almost completely methylated and a slightly yellow nonamethyl derivative could be obtained by distillation but it was found advantageous

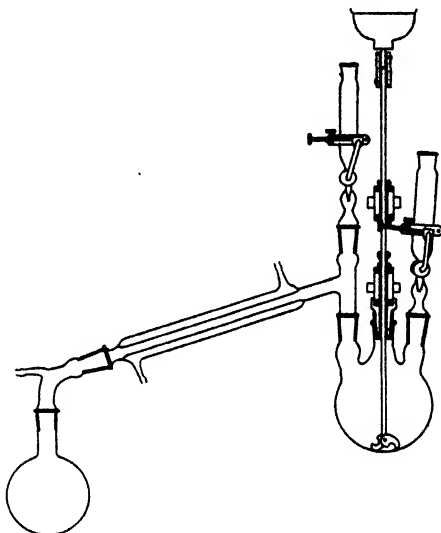


FIG. 1. High speed stirring apparatus for methylation

to remethylate the syrup by the Purdie method. The syrup was dissolved in methyl iodide and 25 gm. of silver oxide were added during 5 hours at 35–40° with stirring. The reaction was allowed to continue for an additional 4 hours. The silver residue was filtered and washed; and the filtrate was evaporated and distilled. B.p. 167–173°, $p = 0.3$ mm. Yield, 26 gm. of a colorless syrup. $n_D^{25} = 1.4542$.

$$[\alpha]_D^{25} = \frac{-0.45^\circ \times 100}{1 \times 9.2} = -4.89^\circ \text{ (in absolute ethanol)}$$

3.497 mg. substance: 39.41 cc. 0.01 N $\text{Na}_2\text{S}_2\text{O}_3$ (methoxy)

4.825 " " : 9.475 mg. CO_2 and 3.905 mg. H_2O

$\text{C}_{21}\text{H}_{48}\text{O}_{11}$. Calculated. C 53.37, H 9.00, OCH_3 59.32

470.3 Found. " 53.55, " 9.05, " 59.08

Hydrolysis and Oxidation of Nonamethyl β -4-Glucosidosorbitol—7 gm. of nonamethyl glucosidosorbitol were dissolved in 90 cc. of 5 per cent hydrochloric acid. The slightly turbid solution was filtered through a thin layer of charcoal and sealed into a bomb tube. This was heated at 100° for 3 hours. The solution was then neutralized with 50 per cent sodium hydroxide. The total volume was 105 cc. 0.5 cc. of this solution was titrated according to Willstätter and was found to equal 11.6 cc. of 0.0135 N sodium thiosulfate. Calculated tetramethyl glucose, 3.3 gm. Found, 3.8 gm.

230 cc. of 0.3 N iodine in potassium iodide were added to the solution which was cooled in an ice water bath. Then 5.5 gm. of sodium hydroxide in 100 cc. of water were added during 3 minutes with stirring. The solution was allowed to stand at 25° for 15 minutes. It was then acidified with sulfuric acid and sulfur dioxide was passed in until the color of iodine disappeared. The solution was then concentrated to a small volume, made alkaline with sodium hydroxide, and extracted with chloroform. The extract was washed with concentrated sodium bisulfite solution and dried. The 1,2,3,5,6-O-pentamethyl sorbitol distilled at 128–133°, $p = 3.0$ mm. Yield 3.3 gm. of a colorless liquid. $n_D^{25} = 1.4439$.

$$[\alpha]_D^{25} = \frac{-0.48^\circ \times 100}{1 \times 4.74} = -10.1^\circ \text{ (in absolute ethanol)}$$

3.341 mg. substance: 39.70 cc. 0.01 N $\text{Na}_2\text{S}_2\text{O}_3$ (methoxy)

5.300 “ “ : 10.110 mg. CO_2 and 4.515 mg. H_2O

$\text{C}_{11}\text{H}_{24}\text{O}_6$. Calculated. C 52.33, H 9.59, OCH_3 61.44

252.2 Found. “ 52.00, “ 9.53, “ 61.44

Another sample had $n_D^{25} = 1.4438$, and

$$[\alpha]_D^{25} = \frac{-1.05^\circ \times 100}{1 \times 11.1} = -9.46^\circ \text{ (in absolute ethanol)}$$

3.201 mg. substance: 37.82 cc. 0.01 N $\text{Na}_2\text{S}_2\text{O}_3$ (methoxy)

6.017 “ “ : 11.510 mg. CO_2 and 5.120 mg. H_2O

Found, C 52.16, H 9.52, OCH_3 61.09

The aqueous, alkaline mother liquor was acidified and extracted with chloroform. The substance distilled at 103–105°, $p = 0.1$ mm. Yield 2.2 gm. $n_D^{25} = 1.4541$.

$$[\alpha]_D^{25} = \frac{+12.74^\circ \times 100}{2 \times 6.03} = +106^\circ \text{ (in absolute ethanol)}$$

6.399 mg. substance: 12.085 mg. CO₂ and 4.300 mg. H₂O

C ₁₀ H ₁₈ O ₄ . Calculated. C 51.29, H 7.75	
234.1	Found. " 51.50, " 7.51

For 2,3,4,6-tetramethyl δ -*D*-gluconolactone, the constants are b.p. 101°, $p = 0.06$ mm., $n_D^{14} = 1.4566$,⁵ and $[\alpha]_D = +101.1^\circ$ (in alcohol, $c = 3.65$).⁶

⁵ Haworth, W. N., Hirst, E. L., and Miller, E. J., *J. Chem. Soc.*, 2436 (1927).

⁶ Charlton, W., Haworth, W. N., and Peat, S., *J. Chem. Soc.*, 89 (1926).

REACTIONS OF DYES WITH CELL SUBSTANCES

IV. QUANTITATIVE COMPARISON OF TISSUE NUCLEI AND EXTRACTED NUCLEOPROTEINS

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One of the most interesting questions confronting the biochemist working with nuclear substances is, what similarities does an extracted nucleoprotein show to the substance existing in the fixed cell nucleus in the form known as chromatin? Two principal points have been investigated in an attempt to answer this question. First, does an extracted nucleoprotein have the same nucleic acid to protein ratio as the substance in the fixed cell nucleus? Second, what is the relation of the so called isoelectric point of the nucleus, as shown by dyes, to the cataphoretic isoelectric point of the extracted substance?

Hammarsten and Hammarsten (1) and Steudel and Takahata (2) have shown that nucleic acid and protein combine in many different ratios when mixed in solution at different pH values. Huiskamp, Bang, and others (see Kossel (3)) have found nucleoproteins of variable compositions when extracting calf thymus glands. In view of these results and others it has been said that no preformed combination of nucleic acid and protein exists in the cell nucleus, the particular nucleoprotein obtained by extraction being the result of the method of extraction and bearing no particular relationship to the nucleoprotein of the chromatin. Nevertheless, it was considered worth while to see whether it was possible to extract nucleoproteins of constant composition from different batches of the same type of tissue, the method of extraction being kept as constant as possible.

Methods of comparing extracted substances with the substances

in the fixed cell nucleus are decidedly limited. Measurements of optical density on cell nuclei, nucleic acid, and nucleoproteins have been employed by Caspersson, Hammarsten, and Hammarsten (4) and Caspersson (5), with the ultraviolet microscope as a tool, and this method is yielding much valuable information.

Combinations of cell nuclei and nucleoproteins with dyes appeared as another possibility of showing similarity or dissimilarity between extracted substance and tissue nucleus. Numerous investigators (6) have dyed tissues with acid and basic dyes to determine the so called isoelectric point of the nucleus and of the cytoplasm. It was believed that some correlation might exist between the isoelectric point found in this manner and the isoelectric point found for the extracted nucleoproteins.

Special methods were developed by which it was possible to note very fine differences in the point of attachment of acid and basic dyes for different tissue elements. These methods soon showed a complete lack of correlation between what was considered as the tissue isoelectric point and the isoelectric point of the extracted nucleoprotein. Investigations on the dyeing of the extracted nucleoproteins also showed a complete lack of correlation with what would have been expected according to the usual protein-dye reactions.

A method of quantitatively determining the amount of basic dye bound by extracted nucleoprotein and tissue nuclear material was devised, based on the work of Bungenberg de Jong and Lens (7). The quantitative relationships found by this method for the extracted nucleoproteins and the tissue nuclei in conjunction with the analytical data obtained on the extracted substances lead to the belief that the extracted nucleoproteins are very similar in their nucleic acid to protein ratio to the substances existing in the fixed cell nucleus in the tissues studied. It is further contended that the attachment of a basic dye to a nucleoprotein is independent of the cataphoretic isoelectric point of the substance, and principally conditioned by the amount of nucleic acid in the nucleoprotein. The methods and discussion of these investigations are given in this paper. The use of the tissue dyeing technique based on these investigations for a number of tissues is given in Paper V of this series.

Methods and Results

The method used to determine the similarity of extracted nucleoprotein and fixed nuclear substances was to compare three types of tissue having nucleoproteins of different nucleic acid content.

The tissues used were Philadelphia No. 1 rat sarcoma, Walker No. 256 rat carcinoma, and rat thymus gland. There were several reasons for using transplantable rat tumor. The tumors were constant in appearance and were very nearly one pure type of tissue when obtained 12 to 14 days after inoculation. The amount of material available was quite large, since the tumors grew to a reasonably large size before necrosis occurred. Thymus tissue was used because it also appeared as a relatively unmixed tissue consisting largely of thymocytes of constant appearance when the glands were obtained from young animals at about 3 months of age.

To determine whether it was possible to obtain nucleoproteins of constant composition from these tissues, four extracts of large batches of each type of tissue were made over a period of 2 months.

The method of extraction was approximately the same in all cases. The freshly removed tissue was freed of adhering fat or other tissue and quickly minced in a Latapie or an embryo extract mincer, depending upon the amount of tissue available from a particular group of rats. This finely minced material was suspended in $2\frac{1}{2}$ times its weight of distilled water in a closed bottle, layered with toluene, and placed in the ice box at 4° . It was allowed to extract with occasional shaking for from 48 to 72 hours. The undissolved tissue was separated from the opalescent milky liquid with cheese-cloth, centrifugation, and filtration through two layers of Whatman No. 5 filter paper on a suction funnel. The filtered liquid was treated with acetic acid to give complete precipitation. This precipitation was found to be complete at pH values between 4.0 and 4.5. The precipitate was either obtained at once, by centrifugation, or allowed to settle overnight at 4° . It was then washed twice with distilled water, resuspended in a volume of water equal to the original amount, and treated with dilute sodium hydroxide to give a pH value between 9 and 10. Solution was complete in all cases but the clear solution was again filtered through two layers of Whatman No. 5 paper. The clear filtrate was again treated with acetic acid to give complete pre-

cipitation at pH 4.0 to 4.5. This precipitate was collected by centrifugation, washed once with water, dehydrated with alcohol and ether, and dried in a vacuum. The average yield of dried nucleoprotein was 3 per cent of the wet weight of the Philadelphia

TABLE I
Nucleoprotein Analyses, Per Cent

Element	Sample No.	Philadelphia No. 1 rat sarcoma	Walker No. 256 rat carcinoma	Rat thymus
Carbon	1	50.30	50.72	49.48
	2	50.62	50.69	49.54
	3	50.04	51.14	49.43
	4	50.07	50.82	48.80
Hydrogen	1	6.36	6.72	6.48
	2	6.42	6.83	6.66
	3	6.33	6.62	6.63
	4	6.39	6.76	6.68
Nitrogen	1	16.07	16.07	17.28
	2	16.28	16.22	16.77
	3	16.34	16.30	17.05
	4	16.45	16.14	17.10
Phosphorus	1	1.07	1.65	2.74
	2	1.06	1.58	3.02
	3	1.07	1.65	2.83
	4	1.04	1.71	2.98
Sulfur	1	1.11	1.42	0.81
	2	1.06	0.99	0.69
	3	1.02	1.07	0.78
	4	1.03	1.15	0.45
Moisture	1	8.65	9.34	7.68
	2	11.66	9.50	8.65
	3	8.84	10.33	8.31
	4	10.30	6.74	7.38
Ash	1	0.50	2.47	0.62
	2	0.63	1.63	0.74
	3	0.53	0.95	0.73
	4	0.50	0.54	0.59

No. 1 tumors, 2.9 per cent of the wet weight of Walker No. 256 tumors, and 7.1 per cent of the wet weight of rat thymus.

Two criteria were used to determine the constancy of the substances obtained. Complete analytical data obtained by careful microanalytical methods were used to determine the nucleic acid

to protein ratio. Isoelectric points, as determined in a micro-cataphoresis cell, were used as the second indication of constancy of the substance obtained.

Since most of these substances are highly hygroscopic, special methods for the determination of the moisture content, for the drying of the samples, and for the weighing under elimination of contact with moist air have been developed by Alber (8). With the use of these special techniques reliable results are obtained in the microdetermination of carbon and hydrogen (9), nitrogen (Dumas method) (9), phosphorus ((10) p. 126), sulfur ((10) p. 116), and moisture (8). The values for the ash content do not represent the true values, since they were obtained by heating the sample in a current of oxygen to a maximum temperature of 650°. Part of the phosphoric acid may be reduced under these conditions to elementary phosphorus and volatilized (11). All the microchemical analyses have been carried out by the microchemical department of the Foundation under the direction of H. K. Alber (12).

Table I gives the analytical results found on the three types of nucleoproteins.

Isoelectric Points

Determinations of the isoelectric points of these nucleoprotein substances were carried out in a cell similar to that described by Bull (13). The Philadelphia No. 1 tumor nucleoproteins were first used in Kolthoff's borax-succinic acid buffers and later checked in Sørensen's citrate buffers. Walker No. 256 and thymus nucleoproteins were tested in citrate buffer in the following manner. 0.1 per cent solutions of protein were made up by dissolving the dry substance in a little water and NaOH with grinding to insure complete wetting, making up to the correct strength at pH 9 to 10, and then adjusting after filtration to a pH of exactly 7 by the addition of a few drops of HCl. The isoelectric point was determined by adding 2 cc. of the 0.1 per cent solution to 25 cc. of buffer solution of 0.02 M concentration. Since the substances were insoluble at pH values near their isoelectric points, it was possible to observe the particles migrate under the low power lens of the microscope. The point on the pH scale at which no migration could be observed was considered to be the isoelec-

60 Dye Reaction on Cell Substances. IV

tric point. Table II gives the isoelectric points found for the twelve samples of nucleoproteins.

The method for determining the points of attachment for acid and basic dyes to the tissue nuclei is described in detail in Paper V of this series (14). Briefly, it consists of immersing the tissue in buffer solution at the same pH as the dye for 15 minutes preliminary to the actual dyeing. After washing, the tissue is dyed for 1 minute in basic dye, then after rinsing, for 1 minute in acid dye. Brief water and alcohol rinses gave finely differentiated slides when buffers at 0.2 pH intervals were used from pH 2 to 7.

TABLE II
Isoelectric Points

Sample No.	Nucleoprotein		
	Rat thymus	Walker No. 256 rat carcinoma	Philadelphia No. 1 rat sarcoma
1	4.25	3.82	4.65
2	4.25-4.30	4.40	4.65
3	4.20-4.25	4.40-4.45	4.60-4.65
4	4.25-4.30	4.40	4.65

Dye Extraction Experiments

For the determination of the amount of dye taken up by the nuclei of the different tissues a special technique was developed. From eight to twelve pieces of tissue cut at 7 microns thickness were mounted on a single slide and dyed with toluidine blue in nearly the same manner as the previous sections used for the determination of the isoelectric points. The dye used was a 0.05 per cent solution of toluidine blue instead of a 0.1 per cent solution, and the sections were dyed for 3 minutes instead of 1 minute. After dyeing, the excess dye was completely rinsed out with several changes of distilled water until no more color could be noted in the solution. The sections were then placed in special extraction cells holding the slide and 4 cc. of liquid. For extraction a 1 per cent solution of thorium nitrate (approximately 0.02 M) was used. This solution completely removed the dye from the nuclei with two extractions of 4 cc. each. The cell was then rinsed with 1 cc. of solution and the volume of the extracted dye solution was

made up to exactly 10 cc. Colorimetric comparisons were then made on the different samples to determine the relations of the

TABLE III
Extraction Experiments with Toluidine Blue
1. *Tissue Comparison*

Tissue	Fixative	Per cent increase in dye, pH 3.5		
		Philadel- phia No. 1 rat sarcoma	Walker No. 256 rat carcinoma	Rat thymus
Tissue	95% alcohol	1	32.2	68.3
"	HgCl ₂	1	19.0	63.3
"	Formaldehyde	1	7.8	
Nucleoprotein	Isoelectric ppt.	1	31.8	49.6

2. *pH*

Tissue	Fixative	Per cent increase in dye at pH				
		2.82	3.2	3.55	3.9	4.3
Philadelphia No. 1	95% alcohol			1	15.5	43.1
" " 1	HgCl ₂ , pH 6			1	17.5	32.8
" " 1	Formaldehyde			1	14.7	24.4
Walker No. 256	95% alcohol		1	18.7	40.4	
" " 256	HgCl ₂ , pH 6		1	16.0	39.6	
" " 256	Formaldehyde		1	20.1	36.2	
Thymus	95% alcohol	1	22.0	35.0		
"	HgCl ₂ , pH 6	1	19.9	29.3		
Nucleoprotein						
Philadelphia No. 1	Isoelectric ppt.			1	14.9	43.9
Walker No. 256	" "		1	24.3	49.3	
Thymus	" "	1	24.2	41.7		

3. *Fixative Comparison*

Tissue	Per cent increase in dye for fixative stain, pH 3.5		
	HgCl ₂	95 per cent alcohol	Formaldehyde
Philadelphia No. 1	1	50.9	73.4
Walker No. 256	1	36.7	63.5

amounts of dye taken up by the different tissues under various conditions.

The sections were rinsed completely free of thorium nitrate solution and redyed, after the usual buffering, by immersion in the 0.05 per cent toluidine blue and then in fast green for counter-staining. After they were mounted under balsam, the area of the sections was measured in the following manner. Each section was projected at a magnification of 10 diameters and the outline of the projected section was drawn on a sheet of paper. The area measurements were then made with a Keuffel and Esser planimeter and the total number of sq. inches of tissue was found by adding the number of sq. inches of all of the sections and dividing by the exact magnification of the projected image. The measurements made in this way were accurate and were considered superior to the commonly used method of cutting out around the projected image and weighing the cut out paper. The sections were also used for nuclear counts.

Table III gives the averaged results of the tissue-dye extraction experiments. In Part 1 of Table III is given the comparison between the amount of dye taken up and released by the three tissues fixed in various solutions and dyed at pH 3.5. Part 2 shows the effect of pH on the amount of dye taken up by the different tissues under various conditions of fixation. Part 3 shows the effect of the different fixatives on two of the tissues when these were dyed at pH 3.5. Fig. 1 shows the pH series of Table III plotted as per cent increase in dye against the pH value.

Extracted nucleoproteins were also dyed with toluidine blue and extracted with thorium nitrate. The procedure in this case was to take 1 cc. of 0.1 per cent protein solution as prepared from the dry substance at pH 7.0. This was precipitated in a 15 cc. centrifuge tube at the isoelectric point by the addition of 5 cc. of citrate buffer solution at the correct pH. The precipitated material was resuspended in citrate buffer of the same pH as the staining solution to be used, and allowed to remain suspended for 15 minutes. After centrifugation it was again suspended, this time in 0.025 per cent buffer solution of the dye and allowed to remain, with shaking, for 8 minutes from the time of first shaking into the dye solution to the time of reaching full speed in the centrifuge. The centrifugation required 2 minutes and then another 2 minutes to stop and pour off the stain and add the first rinse water. The dyed nucleoproteins were washed three times with distilled water

by shaking vigorously with 10 cc. portions and centrifuging to obtain the dyed precipitate. No dye appeared to come off into solution after the third rinse. The dye was then removed from the protein by means of 1 per cent thorium nitrate solution and

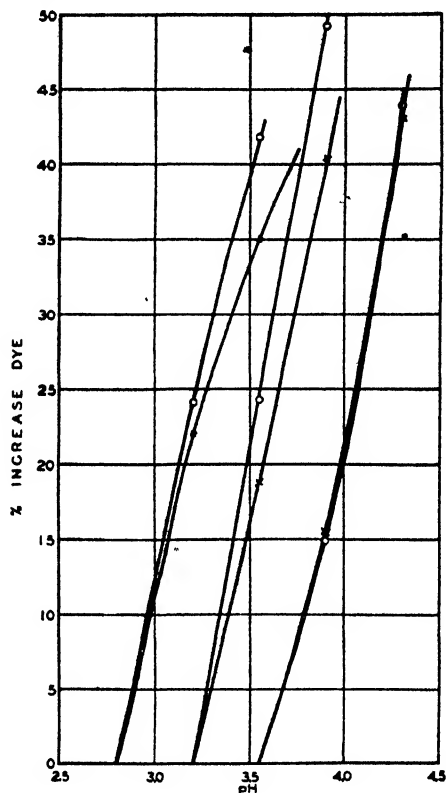


FIG. 1. pH-dye series (Table III, Part 2). O represents nucleoprotein; X, tissue nuclei. The pairs of curves from left to right represent rat thymus, Walker No. 256 rat carcinoma, and Philadelphia No. 1 rat sarcoma, respectively.

two 5 cc. portions were used to remove the dye completely. These were combined and measured colorimetrically.

For a particular experiment all tubes of nucleoprotein were treated as nearly alike as possible and comparisons were made between samples in the same experiment, but not between samples

in different experiments. For example, if a pH-stain series was being run on Philadelphia No. 1 nucleoprotein, three tubes were precipitated, buffered, dyed, washed, and extracted at the same time with the only variable being the pH of buffer and buffered dye solution. If a nucleoprotein comparison was being made, the three tubes contained the different nucleoproteins each of which was precipitated at its correct isoelectric point but each was then prebuffered with equal amounts of the same buffer solution and dyed with equal amounts of the same dye solution, washed, and extracted at the same time. Comparisons were then made between the extracted dye solutions in the same series.

The calculations for the comparison of the amount of dye taken up by the different tissues and nucleoproteins were made in the following manner. The dye solution obtained from the extraction of the Philadelphia No. 1 tissue or nucleoprotein was used as the standard. The colorimetric readings of the extracted dye solutions of the other tissues or nucleoproteins were read against this solution and the results expressed as per cent more dye taken up by Walker No. 256 or thymus tissue or nucleoprotein. The calculation of the amount of nucleic acid in the three nucleoproteins was based on their comparative phosphorus values: 1.06 per cent P for Philadelphia No. 1, 1.65 per cent P for Walker No. 256, and 2.98 per cent P for thymus nucleoproteins; $(1.06/1.65) \times 100 = 64.2$ per cent as much nucleic acid in Philadelphia No. 1 as in Walker No. 256 nucleoprotein or 35.8 per cent more nucleic acid in Walker No. 256; $(1.06/2.98) = 35.6$ per cent as much nucleic acid in Philadelphia No. 1 as in thymus nucleoprotein or 64.4 per cent more nucleic acid in thymus.

DISCUSSION

With the exception of one preparation, the nucleoproteins extracted from the various tissues appear to be uniform in their characteristics. Four samples of Philadelphia No. 1 nucleoprotein have almost identical composition, as shown by the figures in Table I. The nitrogen to phosphorus ratios agree within 3 per cent of the average ratio of 1:15.3. The isoelectric points of the four samples were the same within 0.05 pH unit at the value 4.65.

The four samples of nucleoprotein of Walker No. 256 tumor also

have very similar analytical figures with the exception of the sulfur and ash in Sample 1. The nitrogen to phosphorus ratios also agree within 3 per cent of the average value of 1:9.85. The isoelectric points of three samples were the same within 0.05 pH unit at the value 4.40. The isoelectric point of Sample 1 was 3.8. Electrodialysis of the sample failed to change this value, so it is possible that in this case the increased sulfur was in the form of a non-dissociable sulfuric acid ester having pronounced acidic effect on the compound.

With the less homogeneous tissue, rat thymus gland, the agreement among the four samples prepared is not quite as good. The nitrogen to phosphorus ratio in two of the samples varies as much as 6 per cent from the average of the four samples at a ratio of 1:5.9. The last two preparations agree within 3 per cent. These samples were made from animals of the same age, whereas the first two preparations were made from animals of many different ages. Since the thymus is very susceptible to changes in age, it is believed possible that this may account for the greater difference in nitrogen to phosphorus ratio. The isoelectric points of all four samples agreed within 0.05 pH unit at a pH value of 4.25.

It is not contended that the nucleoproteins as prepared above are completely homogeneous substances. In all preparations it was possible to detect an occasional tiny particle, as observed in the microcataphoresis cell, having a very different isoelectric point from the larger particles of the nucleoprotein under observation. As shown by the pH-stain series (14) the nuclei of different tissue elements varied considerably in their stain-binding capacity. The nucleoproteins of all of the nuclei would probably be extracted equally by the method used. It is believed that it will be possible to fractionate these nucleoprotein preparations in the Tiselius electrophoresis apparatus and thus separate from the tumor preparations fractions representing the lymphocytes, nuclei of the necrotic area, and the mitotic nuclei from the main mass of nucleoprotein representing nuclei of the resting tumor.

The nucleoprotein preparations do strongly indicate that it is possible to obtain substances from the different batches of the same type of tissue having almost exactly the same P:N ratio.

The lack of correlation between the cataphoretic isoelectric points and the points of attachment of acid and basic dyes was

clearly evident after preliminary experiments of the type given in Paper V of this series (14). These experiments showed that the basic dye toluidine blue appeared visually to have dyed the thymocyte nucleus of the rat thymus very strongly at a pH of 3.2. The resting nuclei, constituting the principal nuclear material of the tumor tissues, gave visually complete staining at pH 3.9 for the Walker No. 256 tumor and at pH 4.3 for the Philadelphia No. 1 tumor.

For simple proteins, acting as ampholytes, the isoelectric point represents that point of electrical neutrality in which the charge is zero, due either to lack of ionization of carboxy and amino groups or due to internal neutralization of the charges of ionized groups, according to the zwitter ion theory. In either case, on the acid side of the isoelectric point the protein combines with an acid dye, and on the basic side it combines with a basic dye.

It is apparent that the cataphoretic isoelectric points of the nucleoproteins, as shown in Table II, do not represent points of electrical neutrality toward dyes. Toluidine blue should not dye any of the substances at a pH below 4.3, whereas all of the nuclei were strongly dyed below this point. Table III shows most of the experiments on combination between toluidine blue and tissue nuclei and extracted nucleoproteins carried out at pH values below 4.3. These experiments were possible because not only the tissue nuclei but the extracted nucleoproteins as well showed the strong basic dye attachment at pH values well below the cataphoretic isoelectric points. If the tissue nuclei alone had shown basic dye attachment at pH values below the isoelectric point of the extracted substance, it would have been possible to say that perhaps the nucleoproteins in the nuclei had different nucleic acid to protein ratios and, therefore, different isoelectric points. When the nucleoproteins also were shown to combine basic dye well below the cataphoretic isoelectric point, it was necessary to determine factors other than the outer micelle charge which were responsible for the dyeing of the nucleoproteins.

The dye extraction experiments form this most interesting phase of the work. The experiments of Bungenberg de Jong and Lens (7) show that the bond between negatively charged colloid and basic dye can be broken by the introduction of inorganic ions, the intensity of the action increasing with increasing valence. Na^+ ,

SO_4^- , and La^{+++} cause increasing amounts of basic dye to be released from the negatively charged nucleic acid. The same series of ions was tried with the tissue, nucleoproteins, and nuclei after these had been dyed with toluidine blue. NaCl solution caused very little extraction, Na_2SO_4 somewhat more, and $\text{La}_2(\text{NO}_3)_3$ nearly complete discharge of the dye from the nucleus or nucleoprotein. In view of these results, thorium nitrate was tried and proved to be even more effective than lanthanum, causing a complete removal of the dye from the nucleoproteins and cell nuclei.

The method of calculating the results of the dye extraction experiments brings up a very interesting point. With the alcohol-fixed tissues it was found that when the colorimetric readings of the extracted dye were corrected for the relative areas of the tissues being compared, the results came out in the proportions listed in Part 1 of Table III. That is, when Philadelphia No. 1 tumor tissue was used as the low member of the series, Walker No. 256 tissue was found to have bound 32.2 per cent more dye and thymus to have bound 68.3 per cent more dye than the Philadelphia No. 1 tissue. The calculations for relative per cent of nucleic acid in the extracted nucleoproteins (based on P values) showed 35.7 per cent more nucleic acid in Walker No. 256 than in Philadelphia No. 1 tissue and 64 per cent more nucleic acid in thymus than in Philadelphia No. 1 tissue. These comparative results were even closer than was believed possible, considering the complexity of the method. Nuclear counts were made on the different tissues used for extraction but there was a complete lack of agreement between the nucleic acid values and the dye bound by the nuclei when the number of nuclei rather than the total area of tissue was used as the basis of comparison. The two types of tumor tissue did contain approximately the same number of nuclei, but thymus tissue contained at least twice as many nuclei per equal area as the tumor tissue. On this basis then, the thymus would have taken up about half as much dye as found by the previous method of calculation based on area alone. The nuclei of the two tumor tissues also corresponded quite closely in size but the nuclei of the thymus gland were approximately half the size of those of the tumors.

These results would lead one to believe that the amount of dye

taken up by the alcohol-fixed tissue follows very closely the relative nucleic acid content of the nucleoproteins in the nuclei and that the total volume of nuclear material is very nearly the same in the three tissues examined, although this may be distributed in many more nuclei in one tissue than in another.

The quantitative relationship of dye-binding capacity and nucleic acid content did not hold for HgCl_2 - and formaldehyde-fixed tissue.

Table III also shows the binding of dye by the extracted nucleoproteins. Again, the comparison between the amount of dye bound and then released by the Walker No. 256 as compared with the Philadelphia No. 1 tumor follows very closely the nucleic acid ratio of the two substances. The amount bound by the thymus nucleoprotein was somewhat less than with the tissue and as indicated by the nucleic acid content. The amounts of dye bound varied somewhat in a number of experiments from as low as 45 per cent to as high as 55 per cent more dye bound than that bound by Philadelphia No. 1 nucleoprotein. In no experiment was there complete binding as there was in the tissue. Possibly the nature of the extracted substance prevented complete binding even though the substance was freshly precipitated at its isoelectric point just before dyeing. Even though the amount of dye bound by the extracted substance is about 15 per cent less than indicated by the tissue and by the nucleic acid values of the extracted substance, it is still enough more than in the other substances to warrant the assumption already stated that the extracted substances are very similar even in their dye-binding capacity to the substances as they exist in the tissues.

The pH series of Table III was made to determine the relative binding power of the tissue at different pH values as compared with the binding power of the extracted substance. There are not enough points on the pH scale to consider these values as dye titration curves but even so the three points used for each substance are plotted in Fig. 1. It can be seen that for the Philadelphia No. 1 tumor the extracted substance and tissue are very similar, while with Walker No. 256 tumor and thymus the extracted substance binds somewhat more dye with increasing pH than is bound by the tissue. The values found for the extracted substance were almost within the limits of experimental

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error of 8 per cent for this type of experiment with the extracted nucleoproteins.

The action of fixatives on the quantitative relationship of dye binding is shown in Table III for the two tumor tissues. HgCl_2 is seen to cause a considerable repression of basic dye binding, while formaldehyde causes a considerable increase in the binding

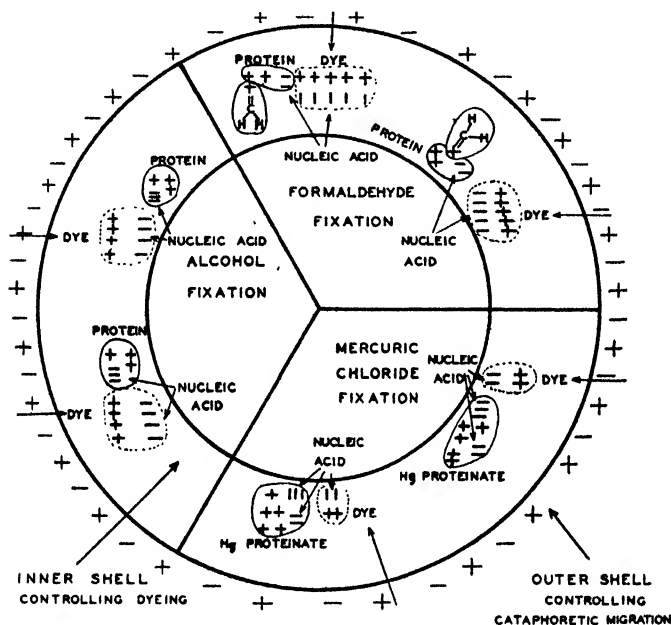


FIG. 2. Diagrammatic representation of dyeing of nucleoproteins as compared with their cataphoretic isoelectric point. The inner shell, controlling dyeing, is predominantly influenced by negatively charged nucleic acid but partially influenced by positive protein charges. The outer shell controls the charge influencing the cataphoretic migration.

of toluidine blue when both are compared with alcohol-fixed tissue. The effect is more pronounced in fixed tissue of the Philadelphia No. 1 than in the Walker No. 256 tissue.

This effect of the fixatives is very interesting, in view of the above results which lead one to believe that the basic dye is largely taken up by the nucleic acid in the nucleoproteins and tissue nuclei. It is not known what effect the fixatives would have on the nucleic

acid portion of the molecule while it was still combined with the protein. The only clear cut effect would be on the protein itself, in which mercuric chloride would combine with the COO^- groups and formaldehyde would combine with the NH_2 groups. Hg^+ should, therefore, reduce combination with the basic dye, while formaldehyde should increase combination owing to repression of basicity within the molecule. The fact that the fixatives work in this manner on the nucleoproteins must mean that the protein is entering into the reaction between the dyestuff and the nucleoprotein. This may be either a direct dye-protein combination or the groups of the protein may influence the availability of the nucleic acid groupings. It seems clear from the results on staining at pH values below the isoelectric point of the extracted nucleoprotein that the ionic atmosphere of the nucleoprotein particle which controls the charge in an electrical field does not control staining. The dye apparently penetrates through this charged field to one caused by the phosphoric acid groups of the nucleic acid. From the effect of the fixatives, however, it is apparent that the charge surrounding these groups is influenced in its reactivity toward dyestuffs by the protein groups associated with it. This supposition is presented diagrammatically in Fig. 2. In this diagram the nucleic acid is represented with many negative charges in order to show the effect of the fixatives. The effect may actually be a greater negative polarity of the phosphoric acid groups of the nucleic acid and not a larger number of electrostatic charges.

SUMMARY

By extraction of nucleoproteins from three different types of rat tissue it has been possible to show that substances of constant composition can be obtained from each type of tissue. The substances obtained in a number of preparations from each type of tissue showed the same nucleic acid to protein ratios and the same isoelectric points. The nucleoproteins from different tissues had considerably different nucleic acid to protein ratios.

Of the three tissues studied, rat sarcoma contained a nucleoprotein having a nucleic acid to protein ratio of 1:15.3. Rat carcinoma contained more nucleic acid in relation to protein and had, therefore, a lower nucleic acid to protein ratio at a value of

1:9.85. Rat thymus had the lowest nucleic acid to protein ratio at a value of 1:5.9.

By experiments on dye-nucleoprotein combination it has been possible to show that the substances extracted from the nuclei of different types of tissue are very similar in their nucleic acid to protein ratio to the nucleoproteins as they exist in the nucleus. In these dye-nucleoprotein experiments it was shown that the dyeing of a tissue nucleus is essentially controlled by the amount of nucleic acid in the nucleoprotein of this nucleus and is independent of the isoelectric charge of the nucleoprotein, as determined by cataphoretic measurement.

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REACTIONS OF DYES WITH CELL SUBSTANCES

V. DIFFERENTIAL BASIC DYE COMBINATION OF TISSUE NUCLEI WITH SPECIAL REFERENCE TO RESTING AND MITOTIC CELLS OF TUMOR TISSUE

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PLATES 1 TO 3

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As a part of the investigation on extracted tumor and thymus nucleoproteins (1) it was necessary to develop a staining method which would show points of acid and basic dye attachment on the different nuclear elements of these tissues. In this investigation it was thought that it might be possible to find some correlation between the so called isoelectric point of tissue nuclei, as shown by acid and basic dye attachment, and the cataphoretic isoelectric point of the extracted nucleoproteins.

Various methods for showing what has been called the isoelectric point of cell nuclei have been developed by a number of investigators. Craig and Wilson (2) used acid and basic dyes and hematoxylin in HCl and acetate buffers in alcohol solution and found this method advantageous for staining differentially tissues of animals, plants, insects, and protozoa. Fautrez (3) and Seki (4) have used toluidine blue in buffer solutions for the differential staining of the cells of the reticulo-endothelial system.

Hammarsten *et al.* (5) dyed nucleic acid and nucleoproteins in buffered dye solutions as well as the red blood cells of lizards. They found protein-nucleic acid mixtures stained as proteins and obtained only surface coloration in many cases. They state that the red blood cells of lizards contained nuclei poor in protein at certain stages of division but rich in protein in other stages. Haynes (6) studied the effect of pH on the staining with thiazine dyes of fixed sections of brain, intestine, bone, spleen, and kidney

and found a general increase in staining intensity from pH 3 to 9. Haynes also used buffers for pretreatment and posttreatment of sections but did not follow this treatment by staining in buffered dye solutions.

Kelley and Miller (7) dyed tissues and nucleoproteins in acid and basic dyes in buffers for comparison of the extracted nucleoproteins with the cell nuclei. They found that the metachromatic staining of nucleoproteins with unmordanted hematoxylin was due to pH effects on the surface of the substances and the differential dyeing of mucoproteins and nucleoproteins with certain basic dyes was independent of pH effects. Laves (8) and Niethardt (9) studied the effect of pH of staining solution on the staining of liver tissue under various conditions. They present a good discussion of the point of staining which can best be considered as the isoelectric point.

Stearn and Stearn (10) have studied intensively the staining of bacterial organisms with buffered dye solutions. They present many experiments which tend to show that dyeing of tissue substances is a chemical phenomenon. Tolstoouhov (11) used acid and basic dyes in combination in 50 per cent alcohol solution to obtain good differential staining of various tissue elements. He studied red blood cells, tumor and kidney tissue and found differential staining of different elements in these tissues. He also studied the effect of the fixative and presented theories regarding the reason for the differential pH staining reactions of the types of nuclei studied.

Most of these methods for showing tissue isoelectric points were tried, but it was found that the point of acid and basic dye attachment was too indefinite to hope to obtain any correlation with an exact measurement such as a cataphoretic isoelectric point. By trying these methods, however, as well as many new staining combinations, it was possible to work out a method giving a sufficient degree of differentiation to make possible an exact study on the nucleoproteins and tissue nuclei. It was found that treatment of the tissue with a buffer solution followed by a short immersion in basic dye in buffer solution and then in acid dye solution gave the most satisfactory results. After each stage the tissues were rinsed briefly in water. After the final water rinse, an alcohol rinse was used to remove excess uncombined dye. Tolstoouhov

(11) and others have stated that the alcohol rinse was not advisable because of its solvent action on the dyes. This was found to be true for a number of dye combinations but in the present method it serves to give even better differentiation than when it is not used. The use of this alcohol rinse did limit the dyes that could be used for this method. Triaminotriphenylmethane dyes such as basic fuchsin or methyl green in combination with acid counter-stains such as fast green and eosin gave dyes of mixed color which were readily soluble in alcohol. It was found that the dyes of the oxazine and thiazine type in combination with either light green or its homologue fast green were essential to satisfy the conditions for the fine differentiation required. Methylene blue, toluidine blue, and thionine as thiazines were all very satisfactory and brilliant. cresyl blue, an oxazine dye, gave fairly good differentiation. Toluidine blue was by all means the best dye found for this method. It was readily soluble in buffer solutions and gave clear, consistent nuclear staining. Thionine, when used in certain buffers, was good, but its lack of solubility was a very limiting factor in its use. Methylene blue was considered to be too unstable chemically to be used over a period of time when constancy of color was desired.

The detailed procedure followed can be given briefly. Acid and basic dyes were made up at a concentration of 0.1 per cent of real dye by dissolving weighed amounts of the dye in 50 cc. portions of buffer solutions. Thionine was soluble in Kolthoff's borax-succinic acid buffers from pH 3 to 5.8, but was not soluble in the Kolthoff borax-phosphate buffers nor in Sørensen's citrate-HCl or NaOH buffers. In one series it was used in the Kolthoff buffers and in another it was used in dilute HCl and in acetate buffers to cover the pH range from pH 2 to 6.

Toluidine blue was soluble in all of the buffers but was used mostly in Sørensen's citrate-HCl and NaOH buffers, since these buffers covered the pH range desired from pH 2.0 to 7.0. Fast green was soluble in all buffers tried. All dyes were of known dye content and purity, as specified by the Commission on Standardization of Biological Stains.

The buffers were made up at 0.2 pH intervals and their pH values determined to ± 0.02 pH by means of a vacuum tube glass electrode apparatus similar to that described by Rosebury (12),

with a General Electric FP 54 vacuum tube. Likewise the dye solutions were tested and their pH value in buffer was found to ± 0.02 pH.

Fixed tissues were cut 7 microns thick from paraffin and passed through xylol and alcohols to distilled water. Individual slides of a series were transferred to 50 cc. of buffer solution in a 35×80 mm. glass shell vial. The slide was allowed to remain in buffer solution of the same pH as the acid and basic dye solution for 15 minutes and was then rinsed in distilled water and transferred to the 0.1 per cent solution of the basic stain where it remained for exactly 1 minute. The sections were then rinsed free of excess basic dye and transferred for 1 minute to the acid dye. After another water rinse the sections were dipped in 95 per cent alcohol until no further running of dye could be noted and then transferred to 100 per cent alcohol, alcohol-xylol, and xylol. All sections in the pH-stain series were mounted under balsam. In most experiments fifteen slides were stained at 0.2 pH intervals covering the range which preliminary experiments showed included the point at which no staining of nuclear material occurred and the point at which no further increase of nuclear staining could be detected microscopically.

In these experiments on the staining of different tissues by the method just given, a number of different fixatives were used to determine their effect on the staining. Preliminary experiments were carried out with alcohol, acetic alcohol containing 5 per cent acetic acid, mercuric chloride at pH 2.0 and 6.0, formaldehyde at pH 2.0 and 6.0, and phosphotungstic acid at pH 2.0 and 6.0.

A comparison microscope was used to enable direct comparison of two sections, stained under different conditions, for location and intensity of color. In most cases better intensity comparisons could be made by using a green contrast filter, such as the Wratten No. B2.

When the observations on staining were made, the points at which basic dye combination with the different cell elements could first be detected were noted, and then the points at which no further increase in intensity of staining was apparent for the different nuclei were found.

The results of the staining experiments on the different tissues and the various types of cell nuclei in these tissues are presented

in Table I. These results represent the values found for a number of series of each tissue with toluidine blue and fast green as the stains.

Discussion of Staining Experiments

The results found in the paper on extracted nucleoproteins showed that the dyeing of nucleoproteins and tissue nuclei followed very closely the nucleic acid content of the nucleoproteins. At a particular pH the tissue or nucleoprotein having the highest nucleic acid content dyed most strongly, while the one with the least nucleic acid took up much less dye at the same pH. From the data of the pH series in this same paper it was possible to show that with increasing pH, increasing amounts of dye combined with all of the substances, but that at any pH along the curve it was still true that the nucleoprotein with higher nucleic acid content was always more strongly dyed. The dyeing of nuclei and nucleoproteins by means of this technique appeared much more nearly like a titration curve of a non-ampholytic substance than a dyeing due to surface electrical charges of an ampholyte. The isoelectric point of the extracted substance may not have been representative of the isoelectric point of the nucleoprotein micelle of the fixed nucleus, but one thing was certain, that the isoelectric charge on the extracted nucleoprotein, as shown by electrical migration, had little influence on the dyeing of the same substance with toluidine blue. Most of the dye extraction experiments were carried out at pH values below the cataphoretic isoelectric point, since it was found that nearly full intensity of staining was reached below this point. Owing to the closeness of the staining reactions of extracted substance and the cell nuclei, it seems justifiable to say that the staining of the tissue nuclei was also independent of a point of electrical balance in any way similar to an actual isoelectric point.

The fact is evident that when a basic dye and an acid dye are used for staining tissue there is a point on the pH scale below which the nuclear material is stained with the acid dye and above which basic dye staining occurs. This point might be considered an isoelectric point, but actually it appeared much more like a dissociation constant for some group in the nucleoprotein. The staining of the nuclei was not a sharply defined point, but began

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with a very faint, scarcely perceptible, staining and continued gradually to a point where visually no increase in intensity of staining could be noted. Probably this first perceptible point of

TABLE I
pH-Stain Series; Toluidine Blue and Fast Green

Tissue	Fixative	Type of nucleus	pH of basic dye solution	
			Point of first staining	Point of complete staining
Philadelphia No. 1 sarcoma	95% alcohol	Lymphocytes	2.4	2.7
		Necrotic area nuclei	2.4	2.7
		Mitotic nuclei	2.7	3.1
		Resting "	3.6	4.3-4.4
	Mercuric chloride, pH 6.0	Lymphocytes	2.4	2.7
		Necrotic area nuclei	2.4	2.7
		Mitotic nuclei	2.7	3.1
		Resting "	6.2	
Walker No. 256 carcinoma	95% alcohol	Lymphocytes	2.4	2.7
		Necrotic area nuclei	2.4	2.7
		Mitotic nuclei	2.6	3.1
		Resting "	3.3	3.8-3.9
	Mercuric chloride, pH 6.0	Lymphocytes	2.4	2.7
		Necrotic area nuclei	2.4	2.7
		Mitotic nuclei	2.6	3.1
		Resting "	3.5	4.4
Thymus (rat)	95% alcohol	Thymocytes	2.5	3.2
	Mercuric chloride, pH 6.0	"	2.8	3.4
Liver	95% alcohol	Lymphocytes	2.4	2.7
		Hepatic cell nuclei	3.5	3.9-4.1
	Mercuric chloride, pH 6.0	Lymphocytes	2.4	3.4
		Hepatic cell nuclei	3.7	4.1-4.3
Spleen	95% alcohol	Lymphocytes, resting and mitotic	2.3	2.6
		White blood cells except above	3.0	3.9
		Reticular cells	3.6	4.0
	Mercuric chloride, pH 6.0	Lymphocytes, resting and mitotic	2.3	2.6
		White blood cells except above	3.0	4.0
		Reticular cells	3.6	4.0

TABLE I—*Concluded*

Tissue	Fixative	Type of nucleus	pH of basic dye solution	
			Point of first staining	Point of complete staining
Kidney	95% alcohol	Tubules of inner medullary zone	3.5	3.7
		Medullary zone tubule nuclei	4.0	5.0
		Glomeruli	4.0	5.0
		Proximal and distal tubule nuclei in cortex	5.8	6.7
	Mercuric chloride, pH 6.0	Medullary nuclei	3.6	Nearly complete
		Glomeruli	3.6	4.8
		Proximal and distal tubule nuclei	4.0	4.8

basic staining is not actually the point of first attachment of the basic dye when one considers the low dissociation constants of the phosphoric acid groups of nucleic acid, but must represent the point at which enough basic dye has entered into the nucleoprotein to overcome the masking effect of the acid dye. The acid dye must also be decreasing in intensity at the same point in the molecule. This must account for the great differential effects in various elements in a single tissue and for the difference between different tissues. The nucleus having the nucleoprotein of greater nucleic acid content will bind more dye at the same pH than one of less nucleic acid content when the time of staining and the concentration of the solution are kept exactly the same. This latter condition is absolutely necessary if the staining of the different types of nuclei is to be interpreted as an indication of the relative amounts of nucleic acid in the nucleoproteins of these nuclei.

In Table I the two most readily detectable points of staining are given, the point of first visual attachment of the dye and the point at which staining appears to be complete. It is difficult to say which point is the most accurate for determining the nucleic acid content, but the point of complete staining appears to be best. For a single tissue it is possible to note this point for differ-

ent elements when they occur in close proximity to one another. For different tissues and unlike regions of the same tissue the comparison microscope allows a very accurate comparison of this point of complete and comparable staining with the different types of nuclei.

It may be seen from Table I that the nuclei of the lymphocytes stain at the lowest pH value and must therefore have a nucleoprotein of the highest nucleic acid content. In comparing the three tissues used in the paper on nucleoprotein preparations, we find the thymocytes of the thymus gland stain at pH 3.2 (Fig. 11), the mitotic nuclei of tumor tissue at 3.1 to 3.2, the resting nuclei of Walker No. 256 tumor tissue at pH 3.8 to 3.9, and the resting nuclei of Philadelphia No. 1 tumor at pH 4.3 to 4.4. In the other tissues studied the lymphocytes likewise showed the lowest point of basic dye attachment, while other elements showed increasingly higher pH values before the basic staining was visually complete.

The differential staining of the resting and mitotic nuclei of tumor tissue forms the most interesting phase of this work. From the reasoning already presented regarding the controlling factors in the staining of tissue nuclei, the fact is evident that the mitotic nucleus has a nucleoprotein of higher nucleic acid content than the resting nucleus in both types of tumor tissue investigated (Figs. 1 to 4, 7, and 8). Several possibilities as to the mechanism of this nucleic acid change can be suggested. More nucleic acid may come into the nucleus from a point of synthesis in the cytoplasm or it may be synthesized in the nucleus. Protein may be split off from the nucleoprotein, leaving a substance relatively richer in nucleic acid. It is even possible that a reorientation of the groups in the nucleoprotein may place more phosphoric acid groups in a position for staining.

In many cases it has been possible to find nuclei having the morphological appearance of a resting nucleus but with the staining intensity of a mitotic nucleus. These nuclei were always found in close association with one or more mitotic nuclei. This would lead to the conclusion that the nucleic acid content of the chromatin material was already increasing before the actual morphological changes of mitosis occurred.

It is interesting to note at this point that Caspersson has already stated that he believes the mitotic nucleus has a higher nucleic

acid content than the resting nucleus, as shown by the greater intensity of the ultraviolet absorption bands of the mitotic nucleus (13). Whether this nucleic acid is free or combined with protein it is not possible to determine with absolute certainty. In these staining experiments the mitotic nucleus definitely has a higher nucleic acid content, but from the point of view of extracted nucleoprotein staining it seems likely that the nucleic acid of the tumor cell nucleus is still attached to protein. The combination must be rather labile, however, in order for the increase to occur as the cell goes through mitosis and for the decrease to occur after the cell returns to the resting stage.

In regard to the possibility that protein may be split off during mitosis there is only one observation that might lead to such a conclusion. That is, that in most of the cells undergoing mitosis there is a distinct increase in the intensity of the acid staining of the cytoplasm of these cells as if the cytoplasm were becoming more basic while the nuclei were becoming acid.

As to the reorientation of the acid groups of the nucleoprotein the only thing that can be noted is that the staining of the nuclei does not appear to be a surface staining phenomenon, but rather there is a definite penetration of dye beneath the outer charged surface. As such, a change in nucleic acid groups to the surface should have little effect.

The effect of fixation on the tissues is not quite as clear as appeared in the quantitative experiments carried out by the dye extraction methods. All of the tissues examined showed considerably less intensity of staining when fixed in mercuric chloride at pH 6.0 than shown by the corresponding alcohol-fixed slides. The resting nuclei of Philadelphia No. 1 and Walker No. 256 tumors showed the most pronounced effect. It was not possible to find any point on the pH scale at which the intensity of staining became equal to that of the alcohol-fixed nuclei. The intensity of staining did appear to increase with higher pH values and with Philadelphia No. 1 tumor at pH 6.2 it was almost equal in intensity to the alcohol-fixed nuclei at pH 4.3. Although there was a pronounced effect of mercuric chloride fixation on the resting nuclei of the tumor tissues, there appeared to be little if any effect on the mitotic nuclei of these same tissues. At pH 3.2, the point of complete staining of mitotic nuclei in alcohol-fixed tumor tissues, the

mercuric chloride-fixed tissue also showed numerous deeply stained mitotic nuclei. At pH 3.8 and 4.3, the points of complete staining of alcohol-fixed resting nuclei in Walker No. 256 and Philadelphia No. 1 tumors, the contrast between mitotic nuclei and resting nuclei is still very pronounced in the mercuric chloride-fixed tissues. This is shown in Figs. 5, 6, 9, and 10. With the thymocytes of rat thymus there was also some inhibition of basic staining but this was not as pronounced as with the resting tumor nuclei. The intensity of staining of the thymocytes was equal to that of the alcohol-fixed nuclei at pH values only 0.4 unit above the point of complete staining after alcohol fixation.

The effect of formaldehyde fixation was even less clear than with mercuric chloride fixation. The nuclear material was more deeply stained than in alcohol-fixed tissue at a corresponding pH, but in the sections examined it was found that fixation of nuclear elements was rather poor and the color was much more diffuse than found with the other fixatives. Because of this diffuse staining, comparisons were difficult between the different sections in a pH-stain series.

In addition to the three tissues investigated in connection with the quantitative dye experiments, a few other tissues from the rat were stained in the differential pH staining series. The tissues so far investigated have been liver, kidney, and spleen. Connective tissue has also been studied where it occurred in connection with the other rat tissues. The complexity of the structure of these tissues renders them unsuitable for a study of the quantitative relations of extracted nucleoproteins and tissue nuclei.

Liver tissue stained in the pH series showed clearly defined hepatic cell nuclei without other deep staining. The full intensity of these nuclei occurred at pH 3.9 to 4.1. Above this point the cytoplasm appeared to show many fine stained granules which it is believed were chromidial bodies. Mercuric chloride-fixed tissue showed somewhat less intense hepatic nuclei at pH 3.9 to 4.1 but gave better contrast owing to the fact that the cytoplasm and granules retained their acid-staining capacity much more strongly in relation to the nuclear staining.

The spleen was much too complex for an exact determination of the point of complete staining for all of the different elements to be made. The different elements appeared to stain progressively

with increasing pH of the stains. The really clear differentiation was notable in the germinal areas of lymphocyte formation. At a pH as low as 2.7, in alcohol-fixed sections, the lymphocytes, both resting and in division, showed very strong basic staining (Fig. 12). Mercuric chloride-fixed sections showed what appeared to be a stronger repression of resting lymphocyte staining than mitotic cell staining, as noted so clearly with tumor tissue. The difference was much less pronounced than with tumor tissue, however, owing perhaps to the relatively smaller percentage of protein in the lymphocyte nucleoproteins. As the pH of the stains increased, there was a progressive increase in the staining of nuclear elements in the spleen tissue. It appeared possible to differentiate between the reticular cells of the white pulp and the various white blood cells other than lymphocytes, but the exact pH values at which these various elements stained completely were not thoroughly investigated.

Of the tissues other than thymus and tumor tissue the kidney was the most interesting. In these experiments the tissues were fixed in two ways: by immersion of the whole organ in the fixing fluid, and by perfusion of the fixing fluid through the glomerular capillaries by injection into the thoracic aorta near where it passes through the diaphragm. The results were exactly the same with both types of fixation and showed very interesting differences between alcohol- and mercuric chloride-fixed tissues.

Alcohol-fixed sections showed no nuclear staining in any regions of the kidney at pH 3.1. At pH 3.7 the collecting tubules in the inner medullary zone were stained fairly strongly. At pH 4.6 the nuclei of the kidney capsule and of the glomeruli were quite intensely stained, while most of the nuclei in the tubules in the medullary zone were very intensely stained (Figs. 13 and 15). The nuclei in the proximal and distal convoluted tubules were not visible in the regions adjacent to the glomeruli in the cortical zone and were scarcely visible anywhere in the cortex (Fig. 13). At pH 5.2 the glomeruli were strongly stained as were also the nuclei of all cells in the medulla. At pH 6.2 the nuclei of the tubules in the cortex were readily visible but not strongly stained. These nuclei of the cortical tubule were finally stained with considerable intensity at a pH of 6.7 and at this point showed with good contrast against the green background of the fast green-

stained cytoplasm (Fig. 14). At pH 7.5 they were even more intensely stained but at this pH the cytoplasm had also stained to a slight extent with the basic dye so the contrast was not as good as in the sections at pH 6.7. In the mercuric chloride-fixed kidney an entirely different staining was found. The differential staining of the different types of nuclei was lacking to a very great extent. At pH 3.3 there was no nuclear staining, as noted in the alcohol-fixed sections, but at pH 3.9 all nuclei were beginning to stain quite strongly. At pH 4.6 in these mercuric chloride-fixed sections the nuclei of the proximal and distal tubules adjacent to the glomeruli were quite strongly stained as were all other nuclei in the sections (Fig. 16). The intensity of all nuclei increased somewhat above this pH but all were of equal intensity.

It is difficult to explain these contradictory facts related to the staining of kidney sections. The rapidity of penetration of the fixing fluid in the perfusion type of fixation should rule out enzymatic or pH changes as a cause of the differential effects noted above. The only explanation considered reasonable is that in the alcohol-fixed sections the proximal and distal tubules contain some strongly basic salts which repress the staining with the basic dye until a high pH is reached. The tubules in the medullary zone were strongly stained in both alcohol- and HgCl_2 -fixed sections at a pH of 4.4, as were also the glomeruli. It appears likely that in the preparation of the mercuric chloride-fixed tissue, in which considerable washing with water was done, these basic salts which repressed staining of the nuclei in the alcohol-fixed sections were washed out.

If such reasoning is valid, the staining procedure has not actually shown a difference in the nucleic acid to protein ratios of different types of cells in the kidney, but only a difference in the basicity of these cells in different regions of the kidney.

SUMMARY

1. A new method of differential staining of tissue nuclear elements has been described. By means of this technique it has been possible to show chemical differences in various types of cell nuclei.

2. The mitotic nucleus of transplantable rat tumor was shown to contain nucleoprotein of higher nucleic acid content than the resting nucleus.

3. Of the tissues studied, lymphocytes were found to contain the most acid nucleoprotein, with other nuclei containing less acid nucleoproteins, in the following order: thymocytes, mitotic tumor nuclei, reticular cell nuclei of spleen, resting cell nuclei of Walker No. 256 tumor, liver nuclei, and resting cell nuclei of Philadelphia No. 1 tumor.

4. The action of fixatives on nuclear staining by the above method has been discussed with special reference to the differential effect of mercuric chloride on the mitotic and resting nuclei of tumor tissues.

5. Kidney tissue fixed in alcohol showed greatly inhibited nuclear staining in the proximal and distal convoluted tubules, while mercuric chloride-fixed tissue showed strong nuclear staining in all the tubules.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Alcohol-fixed Philadelphia No. 1 tumor, stained with toluidine blue and fast green, showing combination of basic dye with mitotic nuclei at pH 3.2. $\times 960$. Wratten No. B2 filter.

FIG. 2. Same as Fig. 1, but showing combination of both resting and mitotic nuclei with basic dye at pH 4.3.

FIG. 3. Same as Fig. 1.

FIG. 4. Same as Fig. 2.

FIG. 5. Philadelphia No. 1 sarcoma, fixed in mercuric chloride at pH 6, stained with toluidine blue and fast green at pH 5.2, showing repression of staining of resting nucleus but not of mitotic nucleus. $\times 960$. No. B2 filter.

FIG. 6. Same as Fig. 5, but at pH 6.2.

PLATE 2

FIG. 7. Walker No. 256 tumor fixed in alcohol, stained in toluidine blue and fast green at pH 3.2. Mitotic nuclei and a few lymphocytes stained. Resting nuclei not stained. $\times 960$. Wratten No. B2 filter.

FIG. 8. Same as Fig. 7, but showing combination of both resting and mitotic nuclei with basic dye at pH 3.9.

FIG. 9. Walker No. 256 carcinoma, fixed in mercuric chloride, showing repression of resting nucleus staining with basic dye at pH 4.3 but not of mitotic nucleus staining. $\times 960$. No. B2 filter.

FIG. 10. Same as Fig. 9, but showing that resting nucleus staining is not as strongly inhibited as it is in Philadelphia No. 1 tumor after mercuric chloride fixation.

FIG. 11. Rat thymus fixed in alcohol, stained in toluidine blue and fast green, showing strong staining of thymocytes at pH 3.2. $\times 487.5$. No. B2 filter.

FIG. 12. Rat spleen fixed in alcohol, stained in toluidine blue and fast green at pH 2.7, showing strong staining of mitotic and resting lymphocytes at this low pH value. $\times 487.5$. No. B2 filter.

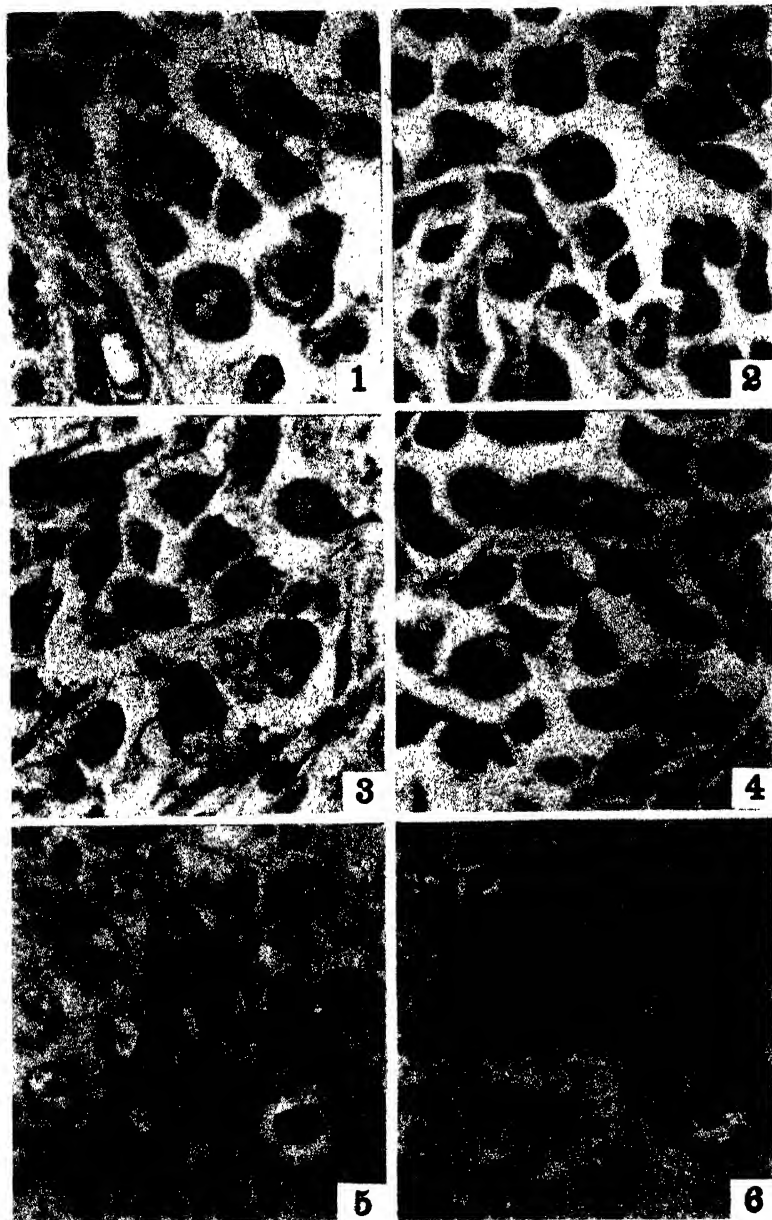
PLATE 3

FIG. 13. Rat kidney fixed in alcohol, stained in toluidine blue and fast green at pH 4.6, showing strong staining of nuclei of the glomerulus but no basic dye staining of nuclei of proximal and distal convoluted tubules. $\times 243.75$. Wratten No. B2 filter.

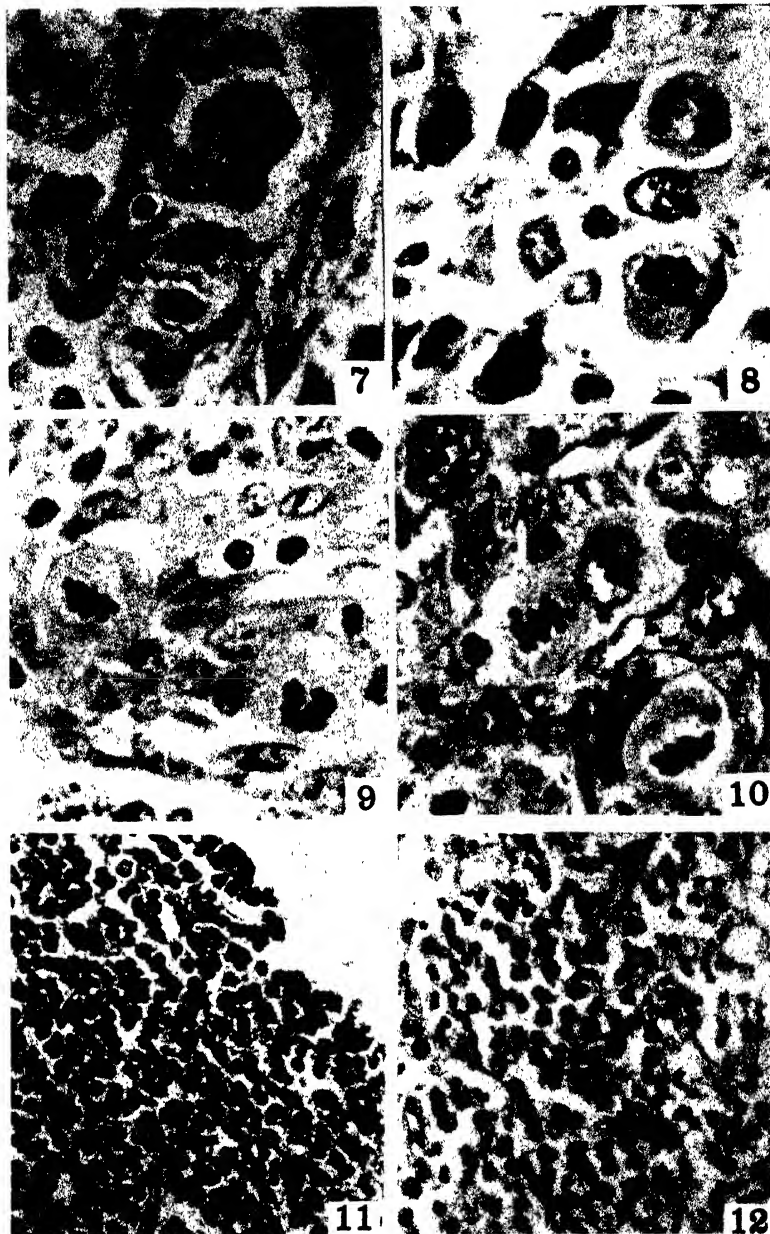
FIG. 14. Similar region of alcohol-fixed kidney, stained in toluidine blue and fast green at pH 6.7, showing nuclei of proximal and distal convoluted tubules stained at this pH. $\times 243.75$. No. B2 filter.

FIG. 15. Alcohol-fixed kidney tissue, showing strong staining of nuclei of medullary region with toluidine blue at pH 4.6, in contrast to lack of staining of nuclei of proximal and distal convoluted tubules of cortex at the same pH. $\times 243.75$. No. B2 filter.

FIG. 16. Mercuric chloride-fixed kidney tissue, stained in toluidine blue and fast green at pH 4.6, showing nuclei of proximal and distal convoluted tubules, stained at this pH after mercuric chloride fixation and subsequent washing out of basic salts which inhibited staining in alcohol-fixed sections. $\times 243.75$. No. B2 filter.



(Kelley: Dye reaction on cell substances. V)



(Kelley: Dye reaction on cell substances. V)



(Kelley: Dye reaction on cell substances. V)

SYNTHESIS OF CYSTINE BY THE ALBINO RAT*

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Within the past few years evidence has been accumulating which is indicative of the probable conversion of methionine to cystine in the animal organism. It has been demonstrated (1, 2) that methionine is as effective as cystine in increasing the growth rate of animals restricted to a low dietary intake of the sulfur-containing amino acids. The feeding of methionine has also been found to make available extra cystine for the detoxication of bromobenzene (3, 4), naphthalene (5), iodoacetic acid (6), and probably also for the detoxication of cholic acid (7). Furthermore, the oral administration of methionine to cystinuric individuals led to an increased excretion of cystine in the urine (8, 9); and either cystine or methionine, given to bile fistula dogs, caused an augmented output of taurocholic acid in the bile (10). More recently, it has been demonstrated (11) that the addition of methionine to the diet of young rats fed a ration known to be deficient in the sulfur-containing amino acids results in an increased hair production and a higher percentage of total sulfur and of cystine in this hair.

Although the results of these contributions strongly suggest that cystine may be replaced by methionine in the diet, probably by virtue of the conversion of methionine to cystine, it is only recently that definite proof has been obtained (12-14) that cystine is entirely dispensable in nutrition when adequate methio-

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† This paper is taken from a portion of a thesis presented by Eliot F. Beach as partial fulfilment of the requirements for the degree of Doctor of Philosophy, Yale University.

nine is available. In the present study, further confirmatory evidence of the non-essential dietary nature of cystine was sought in an investigation of the ability of the animal organism to produce cystine from non-cystine precursors. By feeding a practically cystine-free diet to rats, it has been possible to demonstrate that the animals deposited in their tissues an amount of cystine in excess of that which might be accounted for on the basis of daily cystine intake plus the cystine originally present in the animal at the beginning of the experiment. After the completion of these experiments, the results of a similar investigation were published by Dawbarn (15). The latter investigator observed that the amount of cystine stored in the hair of rats, fed on a basal diet essentially free of cystine and supplemented with methionine, was very much greater than that ingested by the animals, and concluded that evidence had been obtained for the conversion of part of the dietary methionine to cystine.

EXPERIMENTAL

A casein hydrolysate, from which cystine was removed by a modification of the procedure of Vickery and White (16), was used as the source of nitrogen for the experimental diet. This hydrolysate was prepared as follows:

1.5 kilos of casein¹ were hydrolyzed for 18 hours with 450 gm. of granular tin metal and 7 liters of 7.2 N sulfuric acid. At the end of the hydrolysis period the solution was decanted from the tin and divided into five equal portions, each of which was treated according to the following procedure.

The acid concentration was brought to about 2 N by diluting the acid hydrolysate with the correct quantity of distilled water. To the warm (45°) solution was then added a water suspension of cuprous oxide (Kahlbaum's) until a definite excess of the cuprous oxide was present. Efficient mechanical stirring was maintained during the addition of the oxide. The solution was brought to pH 4 to 5 with hot saturated barium hydroxide solution and then chilled for 24 hours. The insoluble material was removed by centrifugation.

The clear supernatants from each of the five portions of the

¹ Technical casein (Lister Brothers, Inc., New York).

hydrolysate, treated as described above, were then combined and saturated with hydrogen sulfide to remove the dissolved copper and tin. The solution was filtered free of the insoluble sulfides and evaporated to about 8 liters on the steam bath *in vacuo*. The sulfuric acid still remaining in solution was removed exactly by precipitation with barium hydroxide. The barium sulfate was removed by filtration, the filtrate concentrated to a small volume on the steam bath *in vacuo*, transferred to an evaporating dish, and evaporated to dryness. The residue was thoroughly dried in a vacuum oven at 70° and ground to a fine powder.

The resulting dry hydrolysate of casein was analyzed for cystine by both the specific Sullivan method (17) and by the Vickery and White procedure (16). Both methods showed no cystine present. It was concluded, therefore, that the removal of cystine had been complete. The cystine-free casein hydrolysate was incorporated in a diet of the following composition: casein hydrolysate 14.7, tryptophane 0.3, Crisco 21, cod liver oil 5, Osborne and Mendel (18) salt mixture 4, and starch 55 per cent.

A group of twelve male rats was placed on the above diet at weaning (21 days of age) and fed the ration *ad libitum*. Each rat received in addition to the above diet a daily supplement of 100 mg. of ryzamin-B (Burroughs Wellcome Company) and 50 mg. of Liver Extract 343 (Eli Lilly and Company). While this quantity of supplement is not optimum, it provided sufficient water-soluble vitamin to permit a fair rate of growth.² This vitamin supplement represents the only possible dietary source of cystine, inasmuch as the casein hydrolysate used in the diet was cystine-free. Analyses of the vitamin preparations employed revealed that the ryzamin-B contained 0.046 per cent organic sulfur and the liver extract 0.654 per cent organic sulfur. Therefore, a daily intake of 100 mg. of ryzamin-B and 50 mg. of Liver Extract 343 represents a daily total organic sulfur intake of 0.373 mg. If all of the organic sulfur in the total vitamin supplement is calculated as cystine, the maximum figure for the daily cystine intake for each animal is only 1.4 mg.

At the end of the feeding period (40 to 44 days) the experimental animals were killed and the gastrointestinal tract and

² Acknowledgment is made to Dr. A. U. Orten for making available her data on the adequacy of this vitamin B supplement.

contents removed and discarded. The pelt and carcass of each animal were hydrolyzed separately in 100 and 150 cc. respectively of 20 per cent hydrochloric acid for 24 hours. The solutions were allowed to cool, so that the hydrolysate could be quantitatively freed, by filtration, from the solid fat and the larger humin particles. The filtrate and washings from each carcass digest were made to a volume of 250 cc. and the hydrolysate and washings from each pelt were made to a volume of 200 cc. with distilled water. The individual hydrolysates were next subjected to a quantitative analysis for cystine by the iodometric titration method developed by Virtue and Lewis (19) for urine and applied more recently by Virtue and Beard (20) to whole rats. After the completion of the analytical work on these animals fed the "cystine-free" diet, Graff, Maculla, and Graff (21) described a modified cuprous oxide cystine method (16) applicable to tissues. This made possible the checking of the cystine values obtained in control groups of animals (see below) by two methods, the iodometric technique and the cuprous oxide procedure. Satisfactory agreement was obtained in the analytical values yielded by these two methods.³

It is of course realized that by both these methods not only cystine but probably also any other substance of a sulfhydryl nature or substances which yield sulfhydryl under the reducing action of the zinc employed in the analytical methods will be determined. For example, homocysteine or homocystine, which might be formed in the metabolism of methionine and deposited in the body tissues, would probably be included in the determinations conducted. Moreover, the iodometric method may be greatly influenced by the presence of other reducing substances in tissues, although the technique devised by Virtue and Lewis (19) has diminished the significance of this source of error. Despite these valid objections, the good agreement between the cystine values found by the two methods of analysis in the instances in which both procedures were employed leads to the belief that the magnitude of the analytical error attributable to

³ Graff, Maculla, and Graff (21) present a choice of a total nitrogen or a total sulfur determination on the thoroughly washed cysteine cuprous mercaptide precipitate. In the present experiments total nitrogen was determined by the micro-Kjeldahl procedure.

non-specificity of chemical reactions involved is not great. The most logical criticism involves the interpretation of the results. It is undoubtedly more logical to consider the analytical values as representing *total sulfhydryl* rather than *total cystine*, even though the latter amino acid represents the major portion of the total sulfhydryl of the organism.⁴

Control animals were needed in order to estimate the average cystine content of weanling rats per gm. of body weight; from the latter value it is possible to calculate the approximate cystine content of each animal in the experimental group at the start of the feeding period. For this purpose, six 21 day-old rats, from six different litters, were selected, having body weights close to those of the experimental animals at the beginning of the feeding period. These six animals were immediately sacrificed and analyzed by the same method which was employed for the experimental group. Furthermore, as already discussed, six additional 21 day-old rats were analyzed by the method of Graff, Maculla, and Graff (21) for comparative purposes.

Results

The results of the analyses of the control animals are given in Table I. The data show that the cystine content of the young rat per gm. of body weight may be quite variable. Furthermore, differences in total body concentration of this amino acid are largely due to variations in the pelt values. Extreme divergence of values is seen in Rats 119 and 124; the other ten rats yielded figures closer to the average. Inasmuch as the cystine content of the pelt reflects the condition of the fur and the quantity of fur present, and since this in turn depends on the nutritional and developmental status of the animal, it is not surprising that such a variability exists in the values obtained for the cystine content, per gm. of body weight, of immature animals of different sizes and from different litters. As already noted, there is satisfactory agreement between the cystine values obtained by the method of Virtue and Lewis (19) and those resulting from the application of the Graff, Maculla, and Graff (21) method of cystine analysis.

⁴ In the form of cystine, cysteine, and glutathione, either free or in the body proteins.

The data obtained on the twelve experimental animals are summarized in Table II. It is of interest to note the good growth exhibited by the animals ingesting the diet which was essentially devoid of cystine. The growth of these animals, therefore, on the diet containing the cystine-free hydrolysate affords confirmation of growth studies (13) on diets known to be cystine-free by virtue of the purity of the individual amino acids fed.

TABLE I
Cystine Content of Rats at Weaning

Rat No.*	Rat weight	Cystine content of pelt		Cystine content of carcass		Total cystine content	
		Total	Per gm. body weight	Total	Per gm. body weight	Total	Per gm. body weight
	gm.	mg.	mg.	mg.	mg.	mg.	mg.
119	44	60.6	1.38	43.8	1.00	104.4	2.38
120	43	78.0	1.81	37.8	0.88	115.8	2.69
121	39	69.4	1.78	35.6	0.91	105.0	2.69
122	41	81.2	1.98	43.8	1.07	125.0	3.05
123	41	80.8	1.97	34.0	0.83	114.8	2.80
124	49	130.0	2.66	43.8	0.89	173.8	3.55
125	40	56.2	1.40	39.2	0.98	95.4	2.38
126	45	72.6	1.61	43.5	0.96	116.1	2.57
127	42	90.8	2.16	49.2	1.17	140.0	3.43
128	37	83.6	2.53	39.8	1.07	123.4	3.60
129	40	77.2	1.93	30.3	0.82	107.5	2.75
130	44	85.8	1.95	37.8	0.86	123.6	2.81
Average.....			1.93		0.95		2.89

* Cystine analyses on Rats 119 to 124 were conducted by the method of Virtue and Lewis (19); Rats 125 to 130, by the method of Graff, Maculla, and Graff (21).

The total cystine intake values of the experimental animals were calculated by multiplying the number of days the diet was consumed by the maximum possible daily intake of this amino acid in the vitamin supplement (*i.e.*, 1.40 mg. of cystine per day). The initial cystine content values were obtained by multiplying the initial body weights by the average cystine per gm. of body weight present in control animals (Table I). The final cystine values were obtained by analyses of the rats. The

minimum cystine synthesis could then be calculated by subtracting the sum of the total initial body cystine content plus total cystine intake from the total quantity of this amino acid present in the animals at the end of the experimental period.

It is interesting that all of the animals except one deposited significantly more cystine than could be accounted for on the basis of that present in the rats at the start of the feeding period and that which might possibly have been fed preformed in the

TABLE II
*Cystine Content and Extent of Cystine Synthesis in Rats Ingesting
Cystine-Low Diet*

Rat No.	Weight of rat		Period on diet	Total cystine intake	Initial cystine content			Final cystine content			Initial body cystine + total cystine intake	Minimum cystine synthesis
	Initial	Final			Pelt	Carcass	Total	Pelt	Carcass	Total		
	gm.	gm.	days	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
106	46	152	40	56	89	44	133	196.8	107.5	304	189	115
107	39	101	40	56	75	37	112	151.2	92.0	243	168	75
108	41	134	40	56	79	39	118	184.4	117.0	301	174	127
109	43	114	42	59	83	41	124	154.0	104.0	258	183	75
111	50	135	41	57	97	47	144	229.6	116.0	336	201	135
112	50	146	40	56	97	47	144	209.6	110.0	320	200	120
113	49	113	42	59	95	46	141	166.4	103.0	269	200	69
114	47	82	42	59	91	45	136	112.8	87.0	200	195	5
115	53	122	41	57	102	50	152	178.3	119.5	298	209	89
116	42	135	44	61	81	40	121	164.5	111.0	276	182	94
117	40	125	44	61	77	38	115	139.0	122.0	261	176	85
118	52	128	40	56	100	49	149	182.0	117.0	299	205	94

vitamin supplement. Rat 114, which grew very poorly, was an exception. The other animals each deposited an average of more than 2 mg. of cystine daily in excess of the amount ingested with the diet. This quantity must have been synthesized from non-cystine precursors. Additional evidence is thus provided that cystine is not a dietary essential for the albino rat provided enough of the cystine precursor, or possible precursors, is available. These experiments, viewed in the light of the results of other investigations and present day information, may be interpreted

as direct proof of the transformation of methionine to cystine in the living organism.

SUMMARY

1. Cystine was quantitatively removed from a casein hydrolysate by precipitation as cysteine cuprous mercaptide.

2. This hydrolysate served as the principal source of nitrogen in a diet which was adequate in all other respects. The only possible dietary source of cystine was the vitamin supplement. The organic sulfur content of the latter supplement, calculated as cystine, permitted a maximum total cystine intake of 1.4 mg. daily.

3. The diet was capable of producing a fair rate of growth in young rats. Furthermore, analyses of the animals revealed that young rats restricted to this diet over a period of approximately 6 weeks usually deposited a quantity of cystine which was considerably in excess of the amount present at the start of the experiment plus the small amount possibly present in the vitamin supplement.

4. It is confirmed that cystine *per se* is not a dietary essential, but may be synthesized from methionine.

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THE METABOLISM OF SULFUR

XXVI. THE METABOLISM OF THE BETAINES OF CYSTINE

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Little is known of the metabolic behavior of the completely methylated derivatives of the amino acids, the betaines, so widely distributed in the tissues of plants and of invertebrate animals (1, 2). The α -betaines, of most common occurrence in nature, are usually stated to be physiologically inactive and to be excreted unchanged in the urine when administered to the higher animals (1-3). Kohlrausch (3), although he accepted this conception of physiological inertness, suggested that after oral administration of the betaine of glycine to rabbits, partial demethylation occurred and trimethylamine was excreted in the urine. The conversion by partial demethylation of betaine to sarcosine as a precursor of creatine has also been suggested (4), although the experimental evidence in support of such a conversion is hardly convincing. The hypothesis that the betaines escape extensive metabolic change is based chiefly upon their isolation from the urine, a procedure fraught with difficulty and hardly quantitative.

The betaine of cystine has recently been synthesized (5) and is available for metabolic study. It is known that the sulfur of N-substituted derivatives of cystine is not oxidized to sulfate and excreted in the urine as readily as is the sulfur of cystine (6-9). Since the amino group of cystine is blocked by methyl groups in the cystine betaine, if cystine betaine were not demethylated, it would be anticipated that after its administration, the sulfur would escape oxidation, and that the sulfur of cystine betaine would constitute a part of the organic sulfur of the urine. If, on the other hand, any extensive demethylation occurred, as suggested by Kohlrausch, the sulfur of the demethylated derivative might be

expected to be oxidized and to appear in the urine in the sulfate sulfur fraction.

If partial demethylation of cystine betaine occurred biologically, a reaction analogous to the suggested conversion of the betaine of glycine to creatine, the formation of N,N'-dimethylcystine should result. The biological behavior of this cystine derivative has not been extensively investigated, since its synthesis has presented difficulties (10). If the theory that N-monomethylamino acids are converted to the corresponding α -keto or α -hydroxy acids is correct (11, 12), N,N'-dimethylcystine should be readily oxidized and utilized. The N-methyl derivatives of homocystine and methionine have been shown to promote growth of the young white rat, when added to a diet known to be deficient in its content of total sulfur-containing amino acids and low in cystine (13). Amino-N-methyltryptophane (14) and amino-N-methylhistidine (15) can be utilized for growth by the young white rat, replacing tryptophane and histidine respectively.¹

In view of these considerations, a study of the metabolic behavior of cystine betaine has been undertaken. The presence of sulfur in the molecule has made possible a method of approach to the study of the behavior of the betaines, other than that of the isolation of the compound under investigation from the urine. The distribution of urinary sulfur after oral and parenteral administration of cystine betaine to the rabbit has been studied. In view of the results obtained with N-methyl derivatives of homocystine and methionine (13), the utilization of cystine betaine for the promotion of growth of the young white rat was investigated. No indications that the betaine of cystine undergoes significant metabolic changes in the organism of these two species were obtained.

Oxidation of Sulfur of the Betaine of Cystine—The general pro-

¹ It is, of course, not permissible to assume, without direct experimental proof, that the α -hydroxy or α -keto derivatives of the naturally occurring amino acids all follow similar metabolic paths. These derivatives of cystine are difficult to prepare in a state sufficiently pure for convincing metabolic study. Similarly, not all the amino-N-methyl derivatives of the amino acids show the same biological behavior. In contrast to the α -N-methyl derivatives of tryptophane, histidine, and methionine, α -monomethyllysine failed to replace lysine for purposes of growth in the organism of the white rat (16).

cedure and analytical methods were those commonly employed in this laboratory in similar studies of the oxidation of sulfur compounds (17). Adult male rabbits weighing approximately 3 kilos served as experimental animals. In order to afford a basis for comparison between the metabolic behavior of cystine and its betaine, the oxidation of the sulfur of cystine was also studied in control experiments with each animal.

The cystine was prepared from hair, and the betaine of cystine according to the procedure of Schubert (5). The specific rotation of the *l*-cystine (1 per cent solution in *N* hydrochloric acid) measured at 25° and with the use of the sodium flame was -210°. Both the cystine and the betaine of cystine were shown to be of satisfactory purity by analyses for nitrogen and sulfur.

The readily soluble betaine was administered in aqueous solution, while the less soluble cystine was converted to the sodium salt by dissolving in the theoretical amount of a solution of sodium carbonate. Both compounds were fed or injected in amounts equivalent to 0.333 gm. of sulfur.

The results are summarized in Table I. The extra sulfur as here presented was obtained by subtracting the average daily urinary sulfur excretions of the control fore and after periods from the sulfur excretions of the various experimental periods. The extra sulfur was also calculated by subtracting the "predicted" sulfur values from the amounts excreted on the days the various experimental substances were administered. The average N:S and N:SO₄S ratios of the urines of the control days were determined. On the basis of these ratios, the predicted sulfur excretions were obtained by dividing the nitrogen values of the experimental days by the appropriate ratio. In this method of calculation, any extra sulfur which might result from a general increase of protein metabolism associated with an increased nitrogen excretion is taken into consideration. In previous studies of sulfur metabolism, we have occasionally observed a stimulation of tissue catabolism and an associated increase in the elimination of both nitrogen and sulfur. No such increased catabolism was observed in the present series and the extra sulfur values calculated by the two methods were in good agreement. For example, the extra sulfur values of Experiment E-1, calculated from the predicted sulfur values, were 0.22 and 0.157 gm. (total and sulfate sulfur re-

spectively), which may be compared with the corresponding values of 0.226 and 0.150 gm. calculated from the average normal urinary sulfur excretion. Similar values for Experiment F-7 were 0.300 and 0.018 gm. respectively, as compared with 0.313 and 0.030 gm.

The data require little comment. After parenteral administration, the sulfur of the betaine of cystine was not oxidized and excreted in the urine as sulfate sulfur, since the extra sulfur of the urine consisted in large part of organic sulfur. Thus, only 5 and

TABLE I

*Distribution of Extra Sulfur in Urine Excreted in 24 Hour Period
Immediately Following Administration of l-Cystine and the
Betaine of l-Cystine*

Extra sulfur values are expressed as per cent of the total sulfur administered, the figures in parentheses showing the percentage distribution of the extra sulfur between sulfate and organic sulfur. The sulfur content of the compounds fed was, in all cases, 0.333 gm. The letters of the experiment number are used to designate the individual experimental animals, while the numeral refers to the number of the experiment. All experiments bearing the same letter were carried out with the same animal.

Experiment No.	Mode of administration	Compounds fed	Extra sulfur excreted		
			Total	Sulfate	Organic
E-1	Oral	l-Cystine	67.6	47.7 (71)	19.9 (29)
F-5	"	"	74	55 (74)	19 (26)
E-4	Subcutaneous	"	76	52 (69)	24 (31)
F-8	"	"	93.4	66 (72)	27.4 (28)
E-2	Oral	Cystine betaine	61.5	19.2 (31)	42.3 (69)
F-6	"	" "	82.5	27 (33)	55.5 (67)
E-3	Subcutaneous	" "	82	4.5 (5)	77.4 (95)
F-7	"	" "	90	5.4 (6)	84.6 (94)

6 per cent of the extra sulfur were excreted as sulfate sulfur after subcutaneous injection of the betaine, percentages which may be contrasted with 69 and 72 per cent of the extra sulfur excreted as urinary sulfate sulfur when cystine was injected similarly into the same animals. These results are comparable to those previously obtained by one of us (L.) in studies with α -phenylureido-cystine (6) and dibenzoylcystine (8) and indicate that blocking the amino group by complete methylation results in a failure of the oxidation of the sulfur of the cystine derivative to sulfate sulfur.

In experiments in which the betaine was fed, there was observed some increase in the excretion of sulfate sulfur, an increase which is believed to be greater than the error of the experimental procedure and to represent oxidation of part of the betaine sulfur. We have discussed previously the possible modification of the metabolic behavior of cystine and related sulfur derivatives (18) by the microflora of the intestine. This effect of the microflora has recently been the subject of direct experimental study (19). We have observed similar slight increases in the excretion of sulfate sulfur after oral administration of substituted derivatives of

TABLE II

Food Consumption and Increase in Weight of Rats Fed Basal (Cystine-Deficient) Diets Supplemented by Cystine and Betaine of Cystine (Litter 8)

The animals of Group VI were females; the other animals, males. The experimental feeding period was of 42 days duration.

Group No.	Rat No.	Supplement to basal diet	Food average daily	Weight initial	Gain	
					Average daily	Per 100 gm. food
			gm.	gm.	gm.	gm.
IV	3214	Cystine	4.8	63	1.38	29.0
	3212	Betaine of cystine	4.8	63	0.37	7.6
V	3225	Cystine	5.2	67	1.37	21.0
	3226	"	5.2	67	1.22	23.8
	3327	Betaine of cystine	5.3	69	0.27	5.2
VI	3237	Cystine	5.2	62	1.19	23.8
	3333	Betaine of cystine	5.2	69	0.21	3.9
	3338	" " "	5.2	63	0.35	6.9

cystine (6, 8). The greater part of the extra sulfur in the present series, as in the previous experiments, is to be found in the organic sulfur fraction of the urine.

Availability of Betaine of Cystine for Growth of Young White Rats—Young rats in litter units were fed a basal diet low in its content of cystine for a period of 14 days. The diet of White (20) was modified to contain 5 per cent instead of 6 per cent of casein (Labco brand, vitamin-free) and 1 per cent of agar. Water-soluble and fat-soluble vitamins were supplied by the separate administration of dried brewers' yeast (approximately 400 mg.)

and cod liver oil (3 drops) daily. During this preliminary period, the animals either failed to gain or showed slight increases in weight. After the preliminary feeding period, the animals were divided into two groups and the paired feeding method employed. One group received cystine as a supplement (0.5 per cent); a second group received the betaine of cystine in amounts equivalent in sulfur content to 0.5 per cent of cystine. With one litter, a third group was continued on the basal diet also. Experiments with three litters, which included twelve rats on the diet containing the betaine and ten rats on the diet containing the cystine supplement, were conducted over a period of 42 days. The results were uniform and may be illustrated by the detailed presentation of the data for a single litter (Table II). The animals which received supplementary cystine showed good growth responses, while their litter mates which received the betaine of cystine as a supplement in place of cystine failed to grow satisfactorily, the rate of growth being similar to that of animals to whose diet (basal) no supplement was added. It is evident that the betaine of cystine is not adequate for the promotion of growth when used as a supplement to a diet known to be low in its content of cystine. These results are comparable to those of Jackson (21) who supplemented a diet deficient in its content of tryptophane with the betaine of this essential amino acid.

SUMMARY

1. After the subcutaneous injection of the betaine of *l*-cystine into rabbits, a study of the distribution of the extra sulfur of urine did not indicate any oxidation of the sulfur of this cystine derivative, since the extra sulfur observed was present in the organic sulfur fraction. The slight increase in oxidized (sulfate) sulfur of the urine observed after oral administration of cystine betaine is believed to be the result of the activity of the microflora of the intestine, which yields products capable of being oxidized to sulfate.

2. Cystine betaine did not function as did cystine in the promotion of growth of young white rats fed a basal diet low in its content of cystine.

3. These results are consistent with the generally accepted belief that the completely methylated derivatives of the α -amino

acids, the α -betaines, are not readily broken down and utilized in the metabolism of the higher animals.

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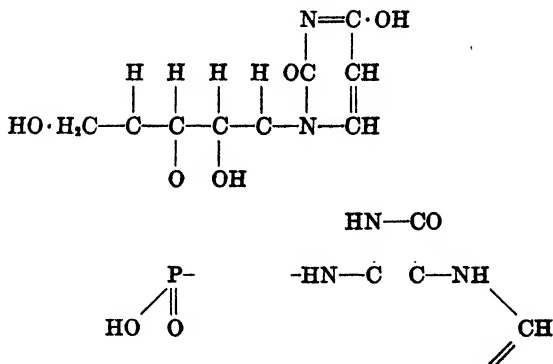
ON THE UNION OF THE NUCLEOTIDES IN RIBO-NUCLEIC ACID

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Bredereck and Richter¹ have announced their discovery of a new product of partial hydrolysis of ribonucleic acid, namely, "guanine-uridylic acid," having the following structure.



The extreme importance of a substance of this structure (in the theory of the mode of union of the individual nucleotides in the tetranucleotide molecule) induced us to repeat the experiments of the above writers, particularly since in one respect their formulation is in disagreement with the observation of Levene and Jacobs² that the primary amino groups of the purine and pyrimidine bases are not substituted in the tetranucleotide. On the basis of this observation there can be no P—N union in the case of cytidine nor,

¹ Brederick, H., and Richter, G., *Ber. chem. Ges.*, **69**, 1129 (1936).

* Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, **43**, 3160 (1910).

perhaps, in the case of uridine. It also seemed to us peculiar that a substance of this alleged composition was not obtainable by Brederick and Richter in crystalline form.

Following the directions of these authors we were able to obtain substances having an elementary composition approaching that of the substances described by them, as shown in Table I.

It can be seen that all these substances have a composition approaching the theoretical for guanino-uridylic acid, but not one is in complete agreement with the theory. An absolutely pure substance could scarcely be expected, when the methods of preparation and the properties to be expected of guanino-uridylic acid are taken into consideration.

In agreement with the statement of Brederick and Richter, the substance forms no precipitate with an ammoniacal solution of

TABLE I
Composition of Various Samples of "Guanine-Uridylic Acid"

	C	H	N	P	Sample No.
$C_{14}H_{16}O_9N_7P$, calculated..	36.75	3.53	21.44	6.79	
Brederick and Richter...	37.20	4.10	21.61	6.61	
Tipson and Levene.....	36.04	4.32	21.79	6.71	541
" " "	36.00	4.16	21.76	6.46	531
" " "	36.15	4.08	23.00	6.65	562
" " "	35.14	4.22	21.92	6.65	804, 1937-38

silver nitrate. In disagreement with these authors, our substances gave in Van Slyke's apparatus an amount of nitrogen equivalent to about 20 per cent of the total nitrogen.

Contrary to what is to be expected from guanino-uridylic acid, our substances always showed a strong orcinol test and, on hydrolysis, a strong reduction of Fehling's solution. These results showed that the substances obtained by the procedure of Brederick and Richter are more complex than was assumed by these authors.

A more detailed analysis of their composition was therefore made. In Table II are given the results of the analysis of four samples, representing four stages of reprecipitation of one parent substance.

It can be seen from these results that all the samples contained

derivatives of *purine nucleosides* in a very considerable proportion. From the sugar and purine contents it also follows that the proportion of purine bases exceeds that of the ribose and hence the samples should contain uncombined purine bases which, however, in the complex mixture are not precipitable by ammoniacal silver solution. Indeed, a sample insoluble in water at 70° (having the composition, C 37.14, H 3.93, N 34.10, P 2.8 per cent) gave a heavy precipitate with an ammoniacal silver solution. On the other hand, a sample (No. 608) having the composition C 37.20, H 4.41, N 24.4, and P 6.25 gave no precipitate, thus showing that the purine nucleotides may inhibit the precipitability of purine bases by ammoniacal silver solution.

TABLE II

Composition of "Guanine-Uridylic Acid" after Successive Reprecipitation

Sample No.	C*	H*	N*	P*	Purine N in air-dry substance	Pyrimidine N in air-dry substance	Ratio of pyrimidine N to purine N	Ribose	Amino N
	per cent	percent	per cent	percent	per cent	per cent		per cent	per cent
540	34.64	4.19	21.50	6.70	17.0	3.3	19.4	17	
↓									
559	35.85	4.09	23.65	6.59				19	
↓									
606	36.71	4.35	22.46	6.44	17.0	2.8	16.5	25	
↓									
612	36.01	4.06	21.47	6.46	19.3*	2.2*	11.3*	26	3.72†

* Substance dried to constant weight (100° at 12 mm.).

† About 20 per cent of the total N.

Sample 612 is one of several on which an amino nitrogen estimation was made in Van Slyke's apparatus, shaking being continued for 30 minutes as required for guanosine.

It was further shown that the purines are present both in the form of free nucleosides and as *nucleotides*, for, from a sample (No. 541) having the composition C 36.04, H 4.32, N 21.79, and P 6.71, both guanosine and barium salts of purine nucleotides could be isolated directly without preliminary hydrolysis. The guanosine was obtained in crude form only, but there was no doubt as to its presence in the mixture.

Attempts to obtain material free from purine nucleosides by

more numerous reprecipitations (by the method of Bredereck and Richter) were unsuccessful, notwithstanding the large quantities of starting material employed (over 3 kilos of nucleic acid). Not infrequently, when the procedures were repeated, the substances with N = 21 per cent yielded material with over 23 per cent of nitrogen, although the values for C, H, and P showed comparatively little change.

Thus no sufficient evidence has as yet been furnished in support of the view that the individual nucleotides are linked through P—N linkages in ribonucleic acid.

EXPERIMENTAL

Preparation of Material—The hydrolysis of the yeast nucleic acid was carried out exactly according to the directions given by Bredereck and Richter.¹ Lots of 100 gm. each were used for individual experiments. The fractions designated by Bredereck and Richter "guanine-uridylic acid," obtained from several experiments, were combined for further purification.

Methods of Analysis—C, H, N, and P determinations were made by the micromethods as commonly practiced in this laboratory by Dr. A. Elek.

Amino nitrogen estimation was made by the micro-Van Slyke method. Inasmuch as the operation lasted 30 minutes, a correction for the blank experiment was used.

Purine nitrogen estimation was made in the following way: The material was hydrolyzed for 4 hours with 2 per cent sulfuric acid (100 ml. for 1 gm.). The purine bases were then precipitated with silver sulfate. The precipitate was decomposed by means of dilute hydrochloric acid and a Kjeldahl nitrogen estimation made on an aliquot part of the filtrate.

Pyrimidine nitrogen was calculated by difference (total N minus purine N).

Ribose was estimated by the Hagedorn-Jensen-Hanes method. The reagent was standardized by means of pure *d*-ribose.

The *analytical results* are given in Tables I and II. In order to save space the details are given below for one sample only; namely, Sample 612 of 1937-38, obtained after four reprecipitations.

6.278 mg. substance:	8.290 mg. CO ₂ and 2.280 mg. H ₂ O
0.100 gm. "	: 15.34 gm. 0.1 N HCl
5.079 mg. "	: 22.600 mg. ammonium phosphomolybdate
13.300 "	: 0.90 cc. N ₂ (758 mm. at 26°)

Found. C 36.01, H 4.06, N 21.47, P 6.46, amino N 3.72

0.5 gm. of substance was hydrolyzed over a free flame with a reflux condenser with 50 cc. of 2 per cent H₂SO₄ for 4 hours. The purine bases were precipitated with silver sulfate, the precipitate decomposed with HCl, neutralized, and the solution diluted to 150 cc., 30 cc. of which contained N 19.23 mg. Hence the total purine N = 96.2, pyrimidine N (107.4 - 96.2) = 11.2 mg. Purine N to pyrimidine N = ca. 9:1. The filtrate from the purine bases, after removal of silver ions, was used for ribose estimation. Found, 0.13 gm. or 26 per cent.

Fractionation of "Guanine-Uridylic Acid"—6.5 gm. of the substance (Sample 541, 1937-38) were suspended in 120 cc. of water, ammonia water was added with constant stirring until all the material dissolved, and the solution was then acidulated by means of acetic acid, the temperature of the solution being maintained at +5°.

Neutral lead acetate was now added to the solution. Only a slight turbidity formed, which was removed by centrifuging with the centrifuge tubes cooled in solid carbon dioxide-acetone mixture. A second precipitate formed on addition of more neutral lead acetate.

This *precipitate* was washed with ice-cold water and suspended in water to which a suspension of barium carbonate was added. Hydrogen sulfide was then passed through the suspension and the lead sulfide was removed by filtration. On standing, a granular precipitate of barium salt formed in the filtrate (Sample 586, 1937-38). It contained no barium phosphate or other mineral salts of phosphoric acid. On concentration of the mother liquor under diminished pressure (the temperature of the water bath not exceeding 35°) a second granular precipitate of barium salt formed. Both precipitates gave a strong orcinol test (characteristic for pentoses, purine nucleosides and nucleotides, but *not* for the pyrimidine derivatives). Both displayed strong reducing power towards Fehling's solution only after hydrolysis.

The substance (Sample 586) had the following composition: N 17.17, P 6.10, Ba 18.00.

Ribose estimation was performed in the same manner as in the previous experiment. The product of hydrolysis of 0.180 gm. of the substance, after removal of purine bases and silver ions and neutralization, was brought to a volume of 15 ml. which consumed 125 ml. of 0.01 N $\text{Na}_2\text{S}_2\text{O}_3$ (1 cc. equivalent to 0.3 mg. of ribose). Total ribose = *ca.* 20 per cent of the barium salt.

The *filtrate* from the lead precipitate, on addition of basic lead acetate and ammonia, gave a second lead salt, which was freed from lead in the usual way and, on concentration, the solution from lead sulfide gave a gelatinous precipitate having the appearance and usual properties of crude guanosine. It contained a considerable proportion of mineral matter but no phosphoric acid ions or conjugated phosphoric acid. Calculated for dry, ash-free substance, the nitrogen content was 24 per cent. (Guanosine, calculated N = 24.73 per cent.) There was not sufficient material to permit of its further purification.

IODINE IN PITUITARY AND SOME OTHER TISSUES

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As long ago as 1886 Rogowitsch (1) found thyroidectomy caused enlargement of the anterior pituitary and increased production of colloid by the cells of the anterior lobe. From these findings he suggested that in thyroid deficiency the hypophysis might act vicariously for the thyroid. Structural resemblances between the pars intermedia and thyroid had been recognized even earlier, especially the presence of colloid material in follicular spaces of the pars intermedia similar to that in the thyroid. And since then, hypertrophy of the pituitary and alterations in its histological appearance have been seen repeatedly in myxedema and other conditions of thyroid deficiency. In fact the interrelation of these two organs has been the subject of hundreds of reports in the last decade, and, while the simple idea of Rogowitsch is no longer held, there is no question that increased thyroid secretion will cause the hypophysis to grow smaller and lack of thyroid function will cause its enlargement. Investigators have consequently been led to look for an accumulation of iodine in the pituitary as evidence of a direct action of thyroid secretion on this gland.

Many attempts were made before 1910 to detect iodine in pituitaries of man (autopsy material), of cattle, and in the glands of thyroidectomized sheep, and these were mostly unsuccessful. The best method available at that time could not detect less than 1 part in 100,000. The more modern techniques are capable of detecting 1 part in 100,000,000 and with these methods the subject has been studied again, for the hypothesis that the thyroid acts directly on the pituitary is an attractive one and there is much evidence to support it. Some of the values found for iodine of various pituitary preparations with the more modern

methods follow: Closs (2), 80 to 190 micrograms per 100 gm. of dried beef pituitary, 40 times the amount in blood; Ruff (3), 400 to 600 micrograms per 100 gm. of dried beef pituitary, 100 times the amount in blood, and similar concentrations in dried pituitary of pig, sheep, and guinea pig; Sturm and Schneeberg (4), 490 micrograms per 100 gm. of fresh dog pituitary; Neufeld (5), 14 micrograms per 100 gm. of dried beef pituitary; while Ohta (6) could find none in rabbit pituitary. Schittenhelm and Eisler (7) found 36 to 70 micrograms per 100 gm. of fresh rabbit dien-cephalon and Noether (8) reported 1.34 *mg.* per 100 gm. of fresh colloid from the pars intermedia of beef pituitary. In human pituitaries, Sturm and Buchholtz (9) found 80 micrograms per 100 gm. and Koppenhoffer (10) reports as much as 13 micrograms of iodine in a single human pituitary. However, he found no iodine in a few pituitaries from patients dying of complications of pregnancy or from certain liver diseases.

The great variability of these observations prompted us, in view of our experience with iodine analysis of foods and tissues, to examine this subject with methods that have been used in this laboratory. Most of the analyses reported here were made with the slightly modified distillation method of Fashena and Trevorrow (11). Because charges of only 10 to 20 gm. can be used in this procedure, it is seriously limited in its application to the estimation of iodine in tissues, at least for amounts normally present. When iodine concentrations are very low and large amounts of material (25 to 50 gm. or more) are obtainable, our closed combustion method (12) is preferred for a first burning and the products of this combustion are then distilled as described in 1937. By either of these methods, 0.1 microgram of iodine (equivalent to about 0.005 cc. of 0.001 *N* thiosulfate) is definitely detectable. The smallest volume of thiosulfate we regard as significant for titration is 0.010 cc. Results obtained by these very different processes, that is by distillation (11) and by combustion (12), agree as shown in Table I.

In Table II will be found the iodine estimations of six pooled specimens of parts of eighteen human pituitaries (the remaining portions were used for histological examinations). In no instance was there a history of iodine medication and no iodine could be detected in the glands. In 40.5 gm. of beef pituitary only 0.7

microgram per 100 gm. of fresh tissue was found, while in about 0.2 gm. of pituitary colloid, which Noether (8) had found to contain more than 1 mg. per cent, no iodine could be found.

In Table III are given analyses of parts of single pituitaries from cases in which there was a history of iodine medication in the last month of life and in three of these iodine was readily

TABLE I
Detection of Iodine by Distillation or Combustion

	Amount	Process	Titration, 0.001 N thiosulfate	Calculated per 100 gm.
	gm.		cc.	cc.
Ox pituitary	15	Distillation	0	Limit of detectability
	40.5	Combustion	0.015	0.040
Rabbit blood	15	Distillation	0.006	0.040
	40	Combustion	0.020	0.050
Alfalfa I	4	Distillation	0.026	0.65
	25	Combustion	0.157	0.63
" II	5.5	Distillation	0.020	0.36
	23	Combustion	0.074	0.32

TABLE II
Iodine of Pooled Pituitaries

Glands	Weight	I ₂ per 100 gm.
	gm.	micrograms
2, human	0.80	<13
3, "	0.90	<11
4, "	1.11	<9
3, "	0.88	<11
3, "	0.74	<14
3, "	1.36	<7
Beef	40.5	0.7
" pituitary colloid	0.2	<50

shown to be present in fairly large amounts. In the remaining three no iodine could be detected.

For comparison spleen, kidney, testis, and ovary of cattle, sheep, and hogs were analyzed. The analyses shown in Table IV were all made in duplicate on 20 gm. samples, except in two analyses of lamb ovaries in which the charges were 5 gm. and

15 gm. Some of the lamb ovaries were infantile, while others contained ripe follicles but no corpora lutea. There appears to be no significant difference, however, between the iodine content of the infantile and maturing lamb ovaries.

The blood iodine of a laboratory sheep, 8 or 10 years old, fed on alfalfa, oats, and lawn grass, was 4 micrograms per cent, while

TABLE III
Iodine in Human Pituitaries

Case No.	Weight analysed	I ₂ per 100 gm.		Remarks
		Wet	Dry	
	gm.	micrograms	micrograms	
374	0.33	<30		15-30 cc. cod liver oil daily for 1 yr. before death
555	0.24	<41		1 dose KI 2 days before death
604	0.30	<33		KI 3 wks. before death
368	0.30	150	660	Dressed with iodoform sponge in last wk. of life
371	0.45	80	390	Same
547	0.28	40	190	100 mg. KI daily for last 2 mos. of life

TABLE IV
Iodine Content of Tissues in Micrograms per 100 Gm.

	Spleen	Kidney	Testis	Ovary
Pig	2	1		0.6
	1	2		2
Beef	0.5	0.5	0.6	2
	1	4		2
Sheep	0.3	4	0.5	5
		2	1	6
		1	2	0.7

that of a young sheep fed on alfalfa and oats was 1.2 micrograms per cent. Blood of four normal rabbits on our stock diet of alfalfa and oats and occasional greens was analyzed and because the diet was low in iodine, 40 cc. samples were used. The values found were 0.9, 1.1, 1.1, and 1.2 micrograms per cent.

According to our analyses, pituitary contains no more iodine

than ovary, testis, kidney, spleen, or blood. Apparent high concentrations of iodine may be due to administration of iodine, a conclusion Wells arrived at in 1910 (13) with the much less sensitive means of determining iodine then in use, or to the presence of important amounts of partly oxidized organic matter in the final stage of the analysis.

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THE DETERMINATION OF SPINAL FLUID PROTEIN WITH THE PHOTOELECTRIC COLORIMETER

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The methods used for the determination of the protein content of the spinal fluid are still far from satisfactory. The micro-Kjeldahl determination on the protein precipitated by tungstic acid or trichloroacetic acid gives accurate results in fluids containing large amounts of protein, but in those with small amounts the precipitation and separation of the proteins present many difficulties. The amount of precipitate is too small to allow for washing without danger of considerable loss in manipulation and unless the precipitate is washed the inclusion of some of the non-protein compounds of the spinal fluids would result in high values.

The turbidimetric method of Denis and Ayer (1) as modified by Ayer, Dailey, and Fremont-Smith (2) is also subject to many errors. The size of the particles and therefore the light absorption can be greatly altered by the slight differences in the amount of agitation or the rate at which the solutions are added. The matching of the solutions in the colorimeter also presents some difficulty, especially for dilute solutions.

Finally the range over which the solutions can be read with an acceptable degree of accuracy is rather limited because of the tendency of the more concentrated solutions to settle out, and this necessitates a repetition of the test after the sample is diluted.

These difficulties have been overcome by the method herein described. The difficulty in reading the solutions in the usual colorimeter have been avoided by the use of the photoelectric colorimeter.¹ By adding a protective colloid the colloidal sus-

¹ We have used the Lange photoelectric colorimeter, but any reliable instrument can be adapted to the method.

pension of the precipitate has been stabilized so that solutions containing as much as 150 mg. of protein can be prepared without danger of the material settling out. As a matter of fact, it is no longer essential to read the solutions within 5 to 30 minutes after they have been prepared, as they will remain stable almost indefinitely. Readings of triplicate samples taken 30 minutes and 30 hours after preparation gave identical results in a series of sixteen specimens. The protective colloid that is added is the gum ghatti introduced by Folin (3) for blood sugar determinations, and later used by Looney (4) for the stabilizing of nesslerized solutions of ammonia.

For the determination 2 ml. of spinal fluid are placed in a small test-tube and 0.5 ml. of 2 per cent gum ghatti solution is added. To this mixture 2.5 ml. of 5 per cent sulfosalicylic acid are added, and the contents of the tube well mixed by rapidly inverting the tube five or six times. When the procedure is properly carried out, there should be no frothing or foaming in the tube. After standing for 5 to 10 minutes the tubes can be read in the photoelectric colorimeter at any time up to 24 hours.

A calibration curve, such as is shown in Fig. 1, must be prepared by diluting a standard solution of blood serum to various concentrations, and obtaining the percentage of light absorption in the photoelectric colorimeter. Spinal fluids containing from 5.0 to 150.0 mg. can be read directly, but more concentrated fluids should be diluted before precipitation. The greatest accuracy is obtained when the protein content is less than 75 mg., at which point the slope of the curve begins to flatten out.

For the standard, pooled serum is diluted 10 times with 15 per cent sodium chloride and filtered. The total nitrogen is determined in an aliquot of this diluted serum containing about 40 to 50 mg. of nitrogen. The non-protein nitrogen of the original serum is determined by the method of Folin and Wu (5) and one-tenth of this value subtracted from the total to obtain the protein nitrogen. The value thus obtained times 6.25 gives the protein content of the standard.

The curve in Fig. 1 was prepared from the readings given by two separate standard stock solutions. Each point represents the mean of three determinations. The value in the triangle at

30 mg. of protein was given by the standard used in the spinal fluid laboratory of the Massachusetts General Hospital.

The precision of the method was tested by determining the protein content of twenty split spinal fluid samples sent to the laboratory under different names. In sixteen of the twenty samples the difference was 1.0 mg. or less. The mean difference between the duplicates was 1.2 mg. with a standard deviation of 0.39 mg.

In Table I the values obtained on the same spinal fluids by the three different methods are given. For the micro-Kjeldahl method only those values were used in which the duplicate analyses showed good agreement. The new method gave only random

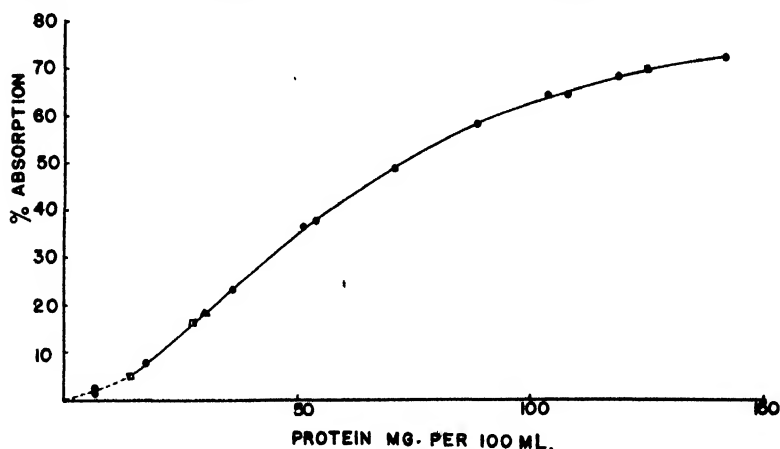


FIG. 1. Spinal fluid protein

variation from these results, yielding a mean difference of approximately 0.7 mg. The determinations by the Ayer, Dailey, and Fremont-Smith method were made in the spinal fluid laboratory of the Massachusetts General Hospital. The figures for the last eight fluids were from split samples sent to the laboratories under different names. It will be noted that for most cases the figures for the new method run higher than those for the Ayer method, the average value being about 3.3 mg. more. However, the values for the two fluids with abnormally high protein content are lower by the new method, and in one of these a micro-Kjeldahl determination gave good agreement with the new method. In

TABLE I
Comparison of Spinal Fluid Protein by Different Methods

Micro-Kjeldahl*		Looney-Walah		Ayer-Dailey-Fremont-Smith	
mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.
49.0	49.0		47.0		
26.0	24.0		21.2		
63.0	60.0		66.8		
15.0	15.0		15.0		
150.0	152.0		152.0		
44.0	45.0		43.0		
33.0	34.0		38.0		
44.0	41.0		40.0		
37.0	36.0		36.0		
43.0	45.0		43.0		
31.0	30.0		25.0		
32.8	33.0		32.0		
53.0	54.0		52.0		
34.0	33.0		31.0		
52.0	54.0		51.0		
177.5			180.0		200.0
			31.0		25.0
			29.2		21.0
			34.5		26.0
			35.0		29.0
			24.0		16.0
			34.5		21.0
			19.0		14.0
			21.5		19.0
			20.2		22.0
			26.8		23.0
			27.2		25.0
		31.0	32.0	27.0	26.0
		19.5	19.0	19.0	18.0
33.6†	33.0	31.0	31.0	20.0	21.0
		31.0	31.5	28.0	29.0
		110.0	109.0	115.0 { ¹¹¹ ₁₂₀	117.0 { ¹²⁰ ₁₁₄
		20.4	21.2	19.0	19.0
		23.3	27.0	23.0	21.0
		19.5	19.0	13.0	13.0

* In this table only those values were used which were consistent in a series of forty duplicate analyses.

† This value is in fair agreement with the value obtained by the new method.

another sample, in which there was a rather large difference between the values obtained by the two methods, an analysis by the Kjeldahl method in duplicate gave a fair agreement with the higher value obtained by the new method. That the difference between the results by the two methods was not due to variations in the standards used was shown by the fact that a determination of their standard by our method gave exactly the same value which they used, 30 mg. per 100 ml.

SUMMARY

A method for the determination of spinal fluid protein with the photoelectric colorimeter is described. The precipitation of protein by sulfosalicylic acid was made in the presence of gum ghatti and yielded a stable colloidal suspension which did not change for 30 hours. Concentrations of proteins from 5.0 to 150.0 mg. can be read without dilution.

We wish to acknowledge our indebtedness to Dr. W. L. Holt and the spinal fluid laboratory of the Massachusetts General Hospital for valuable assistance.

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NOTE ON THE PREPARATION AND PROPERTIES OF HEMOGLOBIN

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In a previous investigation (1) of the equilibrium between oxygen and human hemoglobin it was found that the per cent oxygenation at equal oxygen pressures was very much greater for those preparations which had been purified by an alumina cream treatment than for whole blood or for the product not treated in this way. Since it was our purpose to do some extended work on this equilibrium, we were led to a study of the various methods of preparing this substance and, if possible, to a determination of the factors which give rise to the decreased oxygenating capacity.

The usual criteria of purity, as for example, the ratio of carbon monoxide to dry weight or iron content (2), or the determination of the per cent methemoglobin present, were found quite unsuitable for our purpose. Two samples, which within the limits of experimental error were alike in the above respects, might show a big difference in oxygen saturation. We, therefore, adopted the percentage oxygenation at a given pressure under controlled conditions of temperature and pH as that criterion which would give consistent results.

The existing evidence indicates that foreign substances present in a hemoglobin solution tend to lower the percentage oxygenation. Thus Hill and Wolvekamp (3) have shown that intact red blood corpuscle suspensions show a lower percentage oxygenation than blood solutions. Sidwell, Munch, Barron, and Hogness (1) have demonstrated the depressing effect of salts on the per cent oxy-hemoglobin existing under a given partial pressure of oxygen.

We have therefore assumed that a solution which gives the maximum percentage oxygenation at a given oxygen pressure is purer than solutions which give lower percentages. By the use of this criterion we have been able to determine the efficacy of various methods of purification and select the one which consistently gives the purest hemoglobin.

Method

The percentage saturation was determined spectroscopically as described by Sidwell, Munch, Barron, and Hogness (1). A change in design of the tonometer cells (Stotz, Sidwell, and Hogness (4), and Altschul and Hogness (5)) made it possible to make the determinations with only 3 cc. of solution.

Preparation of Hemoglobin Solutions—All of the operations involved in the following preparations were carried out in a cold room at a temperature between 0° and 3°.

A. Hemoglobin Solution—The horse blood which was used in the preparation was allowed to settle and the erythrocytes were concentrated by decantation. 250 cc. of this suspension of blood cells were washed four times with equal volumes of 0.95 per cent NaCl solution. The suspension, after centrifugation, was treated with 100 cc. of water and 50 cc. of toluene and shaken. After standing a few hours, the solution was centrifuged and the upper fatty layer removed. This treatment was repeated by again adding 50 cc. of toluene and centrifuging. The solution was then dialyzed in a cellophane sac against oxygen-saturated distilled water which was kept flowing continuously for 48 hours. After dialysis, the solution was centrifuged and filtered through a G 5/3 Jena sintered glass filter.

B. Crystallized Hemoglobin Solution—The cells were crystallized according to a modification of the method of Heidelberger (6). 300 cc. of red cells, washed as above, were treated with 100 cc. of toluene and 50 cc. of water. A mixture of 4 parts of carbon dioxide to 1 part of oxygen was passed through the thick suspension for about $\frac{1}{2}$ hour. The vessel was stoppered and left undisturbed overnight to effect crystallization. This process was complete in about 24 hours. After the crystals were centrifuged, the fatty layer was removed and the crystals washed once with cold water. A minimum of saturated sodium carbonate

solution was used to redissolve the crystals. This solution was then centrifuged and dialyzed in a cellophane sac against water through which a mixture of 4 parts of carbon dioxide and 1 part of oxygen was passed. Crystals formed in a few hours. These crystals were again washed and redissolved in sodium carbonate solution, and dialyzed for 48 hours against oxygen-saturated water. As in Preparation A, the final salt-free solution was centrifuged and filtered through a glass filter.

C. Alumina Cream-Treated Solution—The red cells were treated as in Preparation A. After two washings with toluene, the solution was treated with 0.5 volume of γ -aluminum hydroxide suspension prepared according to the method of Willstätter and Kraut (7). The mixture was shaken, centrifuged, and set to dialyze against oxygen-saturated water for 48 hours. When the dialysis was completed, the solution was again treated with aluminum hydroxide suspension, centrifuged, and finally filtered through the sintered glass funnel.

Effect of pH—In order to compare the activity of the three products, it was necessary to correct for any difference of pH of the different solutions. This was done by determining the effect of pH on the percentage saturation of Preparation C (pressure of $O_2 = 1$ mm.) and comparing Preparation C with either A or B at the same pH. The hydrogen ion activity was varied by titrating the dialyzed hemoglobin solution with either 0.025 N HCl or 0.025 N NaOH. Thus the amount of salt introduced was insufficient to affect appreciably the per cent saturation.

The pH curve for Preparation C (Fig. 1) shows no minimum such as was obtained by an extrapolation by Rona and Ylppö (8) and by Ferry and Green (9) on hemoglobin solutions prepared by other methods.

Comparison of Methods of Preparation—Table I shows the relative saturation of solutions of hemoglobin compared with the aluminum hydroxide-treated material. Inasmuch as all the solutions were salt-free, tested at the same temperature (25°), and compared at the same pH, the only factor giving rise to the observed differences must be the state of purity of the hemoglobin, as we have defined it.

From Table I it is obvious that the best of the three methods involves adsorption with aluminum hydroxide. It was found

that crystallization previous to an aluminum hydroxide adsorption did not give a better product.

Nature of Substances Removed by Alumina Cream Treatment—In order to determine the nature of the substances removed by adsorption, a chromatographic analysis (10) was made of a dialyzed blood solution on a column packed with aluminum oxide.

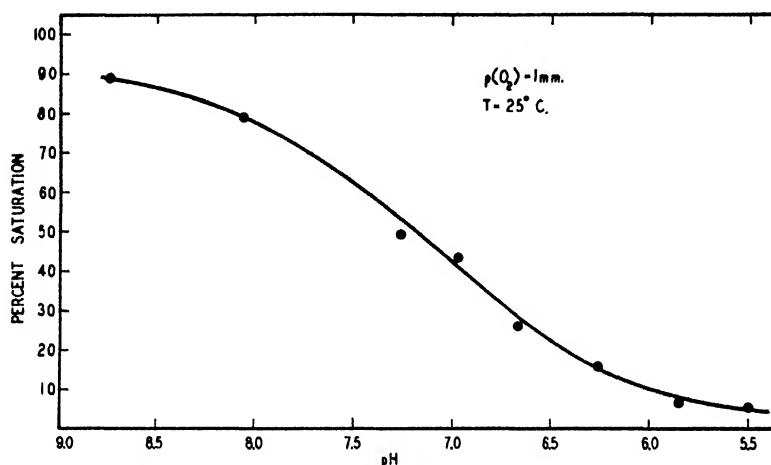


FIG. 1. Effect of pH on per cent oxygenation of hemoglobin (Preparation C).

TABLE I
Comparison of Methods of Purification

Preparation	Comparative saturation (O ₂ pressure = 1 mm.)
C. Aluminum hydroxide-treated solution.....	1
B. Solution from crystallized hemoglobin.....	0.66
A. Hemoglobin solution, fat-free.....	0.30

Two zones of color, both red, were seen after long washing with distilled water. This indicates that one colored constituent of the blood was more firmly adsorbed than the other. A similar observation was made with the alumina cream as the adsorbent. A dialyzed blood solution was treated with γ -aluminum hydroxide and centrifuged. The precipitate, colored deep red, was washed

five times with distilled water. A considerable amount of color was removed by the washings. After the fifth washing, the wash water had practically no color, although the alumina precipitate was still colored pink. The colored substance was then eluted either with neutral phosphate buffer solution or a basic elution mixture used in the purification of yellow ferment (11). Practically all the color was removed by this treatment.

The eluate thus obtained had some activity when tested for "free" oxygen content, although the ratio of oxygen content to dry weight indicated that a considerable amount of the colored substance present was either inactive hemoglobin (12) or methemoglobin. Both the visible and ultraviolet spectra of this eluate showed the characteristic peaks of hemoglobin. Upon dialysis against oxygen-saturated water, a brownish precipitate

TABLE II

Effect of Impurities on Percentage Saturation of Hemoglobin with Oxygen

Type of solution	Comparative saturation (O ₂ pressure = 1 mm.)
Purified hemoglobin.....	1
Hemoglobin + albumin (4.5 mg. albumin per cc.).....	0.8
" + sucrose (0.36 gm. sucrose per cc.).....	0.94
" + urea (0.37 gm. urea per cc.).....	0
" + eluate (1 mg. eluate per cc.).....	0.7

settled out. It was impossible to obtain a value for the percentage saturation with oxygen for the clear filtrate, inasmuch as the removal of oxygen from the solution by washing with nitrogen caused decomposition and methemoglobin formation, and rendered it impossible by subsequent addition of oxygen to bring the substance back to its original state.

Effect of Impurities on Percentage Oxygenation—A number of different substances were added to purified hemoglobin (Preparation C) to determine their effects on the percentage oxygenation. The results represented in Fig. 1 were used in adjusting the pH values so that all comparative data could be given for the same pH (Table II).

We also determined spectrophotometrically the amount of methemoglobin in solution and found that this substance, up to about

40 per cent of the total hemoglobin, has little effect on the percentage saturation of the ferrous form provided that the amount of methemoglobin is constant for the duration of the determination.

All other impurities lower the percentage oxygenation. The eluate apparently contains some substance which has a specific effect on the saturation value.

We have confirmed the statement of Steinhardt (13) that hemoglobin dissolved in 6 M urea still shows the bands of reduced hemoglobin and oxyhemoglobin. This solution, however, acts similarly to the eluate described above, in that it decomposes when washed with nitrogen gas in the process of removing oxygen.

DISCUSSION

For a partial pressure of oxygen equal to 5 mm., a pH of 7.0, and at 25°, 81 per cent saturation has been obtained. This is the same value that was obtained by Sidwell, Munch, Barron, and Hogness (1) for human hemoglobin prepared by treatment with alumina cream and determined under identical conditions. In this respect horse and human hemoglobin are alike. Oxyhemoglobin crystals obtained from horse blood after the alumina cream treatment took the form of long thin needles, while those obtained from the solution without this treatment were in the form of plates. No attempt was made to determine the crystallographic axes, nor have we attempted to obtain crystals from human blood treated in this way.

The fact that two distinct layers were obtained on the Tswett column suggests the possibility of two forms of hemoglobin (14). In the presence of air these two layers when eluted with phosphate buffer were identical spectroscopically. Unfortunately we were not able to determine the capacity of the upper layer to take up oxygen. When the oxygen was removed from the eluted solution by gentle washing with nitrogen, the hemoglobin underwent a rapid irreversible change. We were unable to determine whether the top layer on the column was a different form of hemoglobin or merely hemoglobin adsorbed by or together with some other substance.

The question may be raised as to how we are to define pure hemoglobin. In the erythrocytes, hemoglobin is either in a different form from that obtained by our method of preparation

(C) or it is associated with some other substance. From the biological point of view hemoglobin, as it exists in the cell, is of greater significance, while from the chemical view-point, this "purified" form may very probably be of greater interest.

SUMMARY

1. Purification of horse hemoglobin by adsorption on γ -aluminum hydroxide has been shown to yield samples of hemoglobin which give the highest percentage saturation with oxygen.

2. The effect of pH on the percentage saturation at 1 mm. of oxygen pressure has been determined for the range of pH 5.5 to 8.7. No minimum has been observed in that range.

3. The effect of various substances upon the percentage saturation has been determined.

4. The possibility of the existence of two forms of hemoglobin is considered in the discussion of the experimental results.

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STUDIES ON THE MERCAPTURIC ACID SYNTHESIS IN ANIMALS

X. GLUTATHIONE IN RELATION TO GROWTH OF RATS ON A LOW CASEIN DIET WHICH CONTAINED BROMOBENZENE AND NAPHTHALENE*

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In Paper VIII of this series (1) we reported that in contrast to *l*-cystine and *dl*-methionine glutathione does not augment the synthesis of mercapturic acids in the rat. Data were also obtained to indicate that the cysteine and the acetyl group of the mercapturic acids were not derived necessarily from glutathione (1). These results offered no evidence that cystine or cysteine is liberated from glutathione during metabolism in the rat.

Dyer and du Vigneaud (2) reported results which indicated the possibility that cystine or cysteine may be liberated from glutathione during metabolism, inasmuch as cystine-deficient rats resumed growing when glutathione was administered to them either orally or *sub cutem*. We felt, therefore, that the ingestion of naphthalene and bromobenzene might possibly modify the normal metabolism of glutathione in the rat in such a way as to prevent the liberation from it of cystine or cysteine. Should this be true, it would then account perhaps for the inefficiency of glutathione in augmenting the synthesis of mercapturic acids in the rat. On the other hand, the demonstration that glutathione, when added to a diet which contained bromobenzene or naphthalene, promptly overcomes the deficiency in rats induced by these hydrocarbons would strongly suggest that the metabolic behavior of glutathione in the rat is not altered by the presence

* This paper was presented before the meeting of the American Society of Biological Chemists at Baltimore, April, 1938.

of bromobenzene or naphthalene in the diet. Such a study would offer support to the inference that the growth-promoting power of glutathione, under the experimental conditions, is not necessarily due to the liberation from the tripeptide of cystine or cysteine during metabolism.

Indeed, Dyer and du Vigneaud (2) have pointed out "that the question still obtains in these studies, as in others of this nature, as to whether the utilization of the supplement is due to its conversion in the body to the deficient factor or is only a matter of a sparing action for the small amount of cystine or methionine which is present in the diet, a question so amply discussed by Jackson and Block [(3)]. From this standpoint a sparing action of the glutathione in this particular study could mean that the supplement might be used as such without hydrolysis for certain purposes, and that the small amount of cystine and methionine in the diet thus spared might serve for the synthesis of new protein in the tissues and for other anabolic purposes."

A study of the utilization of glutathione by the rat on a low casein diet which contained bromobenzene or naphthalene was therefore undertaken. In every case it was found that glutathione supported the growth of rats under these conditions.

In the light of our previous data (1) which indicated the failure of glutathione to augment the synthesis of mercapturic acids, the results reported below suggest the utilization of the tripeptide by the rat without necessarily a preliminary hydrolysis of glutathione to yield cystine or cysteine. In this connection it is of interest to note that the administration of bromobenzene (4) or naphthalene (5) to animals leads to a marked decrease in glutathione content of certain tissues, particularly that of the liver and the eye lens. Inasmuch as no evidence was obtained to indicate the direct utilization of glutathione for detoxication purposes to yield mercapturic acids (1), the reports mentioned above (4, 5) suggest that the utilization of cysteine for the detoxication purposes, particularly the formation of mercapturic acids, may lead to a diminished elaboration of glutathione in the tissues. The depleted stores of glutathione in the tissues of deficient animals may be restored by the administration of cystine, cysteine, methionine, or glutathione, the latter being utilized by the animal apparently without hydrolysis to yield cystine or cysteine. A study of the relationship between the glutathione content of the

tissues of animals which received bromobenzene or naphthalene and the dietary glutathione of these animals might perhaps yield information of value to this problem.

From the foregoing discussion it does not follow by any means that the decrease in the glutathione content of the tissues of animals which were fed bromobenzene or naphthalene is the only possible change which is induced by the hydrocarbons in the organism. Neither do our data exclude the possibility of cleavage of glutathione into its constituent amino acids under certain other conditions. Although certain enzymatic studies *in vitro* (6) indicated that no appreciable amount of cysteine can be liberated from glutathione in the intestinal tract, it has been shown recently that the isolated kidney of the rat splits the tripeptide to yield cystine, glutamic acid, and glycine (7).

EXPERIMENTAL

The diet which we used (originally devised by White and Jackson (8)) had the following percentage composition: casein¹ 6, salt mixture (Osborne and Mendel (9)) 4, corn-starch 50, sucrose 15, Crisco 25. In addition, each rat received 400 mg. of yeast powder (Northwestern Yeast Company) and 100 mg. of cod liver oil daily. This diet is referred to in Table I as Diet C-6. Glutathione and *l*-cystine were administered either mixed with food or separately from the diet incorporated in the yeast and cod liver oil supplement which was fed in two equal portions 6 hours apart. The general procedure of the experiments was the same as that used previously (8, 10). The data shown in Table I are representative of the experiments obtained with two litters of albino rats which were 26 days old.

The glutathione and cystine were analytically pure. Bromobenzene was redistilled and naphthalene resublimed before use.

The data in Table I show that the growth of rats which were maintained on Diet C-6 was checked when bromobenzene or naphthalene was incorporated in the diet, and was resumed upon the administration of glutathione to rats which received the supplemented diet. The effectiveness of glutathione in promoting the growth of rats was apparently the same as that of an equivalent amount of *l*-cystine.

It will be observed from Table I that the rat required consider-

¹ Casein 453, Casein Company of America, Inc.

TABLE I

Effect of Glutathione and Cystine on Growth of Rats on Diet C-6 Which Contained Bromobenzene or Naphthalene

The data for each rat are representative of experiments with four animals.

Rat No. and sex	Bromo-benzene per 100 gm. diet	Naphthalene per 100 gm. diet	Glutathione		Cystine		Food intake per day	Initial weight	Total gain	Days on diet
			Per 100 gm. diet	Per day	Per 100 gm. diet	Per day				
	mg.	mg.	mg.	mg.	mg.	mg.	gm.	gm.	gm.	
26 ♂							6.1	46	+33	54
	1000						5.6	79	-2	14
	1000		320				5.8	77	+19	27
	1000						5.2	96	-5	14
							6.9	91	+7	10
	1000						6.0	98	-5	10
	1000				120		7.8	93	+8	10
27 ♀	1000			32			7.7	111	+9	10
							5.3	52	+34	54
	1000		320				5.0	86	-5	14
	1000						6.0	81	+23	27
	1000						5.3	104	-11	14
	1000			32			6.4	93	+17	20
	1000					12	6.7	110	+16	20
28 ♂							6.8	126	+13	14
		250					5.0	52	+26	54
		250	320				5.0	78	-10	14
		500	320				6.0	68	+25	27
		500					5.2	93	-8	14
		500			120		5.6	85	0	14
		500			240		6.7	85	+13	14
29 ♀		500	640				6.9	98	+12	14
		500		64			7.2	110	+16	14
		500					7.4	126	+14	14
						24	5.1	43	+32	54
	250						4.3	75	-3	14
	250	320					5.7	72	+21	27
	250			32			6.2	93	+17	14
34 ♂	250					12	6.2	110	+16	14
	500	320					6.0	126	0	14
	500	640					6.9	126	+10	14
	500	320					6.5	136	-1	14
							6.9	54	+100	122

ably more glutathione or cystine to overcome the deficiency induced by 0.5 per cent naphthalene in the diet than to overcome that induced by 1.0 per cent bromobenzene in the same diet.

Several possibilities may account for this observation, a few of which may be mentioned. As we reported previously (1), the efficiency of the rat to detoxicate naphthalene or bromobenzene varies inversely with the concentration of the hydrocarbons in the diet. Assuming that the relative toxicity of the two hydrocarbons to the rat is the same, it is possible that more mercapturic acid is synthesized by the rat from 0.5 per cent naphthalene in the diet than from 1.0 per cent of bromobenzene in the same diet. It is conceivable also that under comparable dietary conditions more organic sulfur is utilized by the rat to detoxicate naphthalene than is involved in the detoxication of an equivalent amount of bromobenzene. The detoxication product which involves a loss of organic sulfur to the rat may not be necessarily mercapturic acid exclusively. That the mercapturic acid which is excreted in the urine does not represent the entire amount of the acid which was synthesized by the rat from naphthalene is a possibility which also cannot be dismissed *a priori*. It is possible that the mercapturic acid, once synthesized by the rat, undergoes further catabolic changes and is excreted in the urine in a form which is not measurable by the method for the determination of mercapturic acids in urine. Earlier workers have considered the possibility of oxidation of mercapturic acids in the animal body. Sherwin and coworkers (11) presented data to show that *p*-bromophenylmercapturic acid is oxidized to a certain extent in the organism of the rabbit and the dog to yield ethereal sulfates and inorganic sulfates. Coombs and Hele in experiments with dogs and pigs (12) could not, however, confirm this claim. Consideration of the structural differences of mercapturic acids and the corresponding ethereal sulfates precludes the possibility of direct oxidation of the former into the latter. König's studies *in vitro* (13) suggest that mercapturic acids on oxidation may yield corresponding sulfonic acids. Further work along these lines seems necessary.

SUMMARY

1. The growth of rats is checked when bromobenzene or naphthalene is added to a diet and is resumed when glutathione or an equivalent amount of cystine is fed in addition to the supplemented diet.

2. In the light of our previous studies on the rôle of glutathione

in the synthesis of mercapturic acids in the rat, the present data seem to indicate that the promotion of growth by glutathione in the rat is not necessarily due to the liberation from the tripeptide of cystine or cysteine, but perhaps is a result of the utilization of glutathione as such and of the sparing action of the tripeptide on that dietary cystine and methionine which are used by the rat for the synthesis of new protein in the tissues and for other anabolic purposes.

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THE INFRA-RED ABSORPTION SPECTRA OF AMINO ACIDS

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Studies of molecular structure through the medium of infra-red spectroscopy become less productive as the type of molecule under consideration becomes more complex. This method of attack cannot be expected to yield very extensive information in the case of molecules possessing the complexity (large size and lack of symmetry) of the amino acids. Definite knowledge of a more restricted sort, however, can very often be obtained through interpretation of special features of the spectra of such compounds. For example, the behavior of absorption bands which can be ascribed to certain subgroups of atoms such as the carbonyl, hydroxyl, or amino group can sometimes be correlated with the structure surrounding these groups. Again, mere differences in spectra can under certain circumstances furnish conclusive evidence that compound formation between molecules has taken place.

Very little attention has been given to the amino acids by workers in the field of infra-red spectroscopy. Heintz (1) has observed the spectra of a few amino acids in the range from 1 to 9 μ . The spectra of the stereoisomers of cystine in the range from 3 to 24 μ have been recorded by Wright (2). In view of the importance of the amino acids from a biological standpoint it appeared worth while to extend the investigation to a larger number of compounds of this class both to record the infra-red spectra, as physical properties, and to obtain structural information.

The infra-red spectrograph (3, 4) and the method of operation were those used in the earlier investigation (2). Samples were observed in the form of thin, uniform layers of the finely powdered crystals, containing about 1 mg. per sq. cm. of layer area. The method of producing a layer was to allow the powder to settle out

of absolute alcohol upon a plate of crystalline KBr. The alcohol was then removed by evaporation.

The amino acid samples with the exception of serine and the glutamic acid isomers were obtained from the Eastman laboratories, Hoffmann-La Roche, and the University of Illinois. Samples of the glutamic acids were obtained from Amino Acid Manufactures through the courtesy of Dr. M. S. Dunn of the University of California at Los Angeles. Dr. H. B. Lewis kindly supplied samples of *l*-glutamic acid and *dl*-serine.

Tables I and II present the observed spectra in the form of lists of the wave numbers of the absorption bands with rough estimates of the corresponding intensities expressed as per cent of radiation absorbed.

Comparison of the spectra of the different amino acids brings out at once the resemblance which is to be expected from the similarity of the compounds. This resemblance of the spectra does not, however, extend beyond a general characterization. As a matter of noteworthy fact the spectra of the individual amino acids are sufficiently different to serve as a means of identifying the compounds.

The spectra recorded in Tables I and II have been arranged so that comparisons can be made between the optically active and racemic forms of five amino acids. In each case the spectral differences reveal unmistakably that the racemic forms, which have been obtained by crystallization from solution, are compounds and not mere physical mixtures of the *l* and *d* crystals. This will be evident from the following statements: The optically active, *l* and *d* isomers of the same amino acid must have identical infra-red spectra, since the molecules and the crystals of one form are mirror images of those of the other. A structural difference of this kind has no influence either upon the frequencies of mutual vibration of the atoms or upon the associated intensities of absorption. A mechanical mixture made by pulverizing together equal amounts of the *l* and *d* crystals of the same amino acid therefore has a spectrum identical with that of either form taken separately. These facts have been actually demonstrated in the case of the isomers of cystine (2). If now the spectrum of the racemic form of an amino acid differs in any way from that of the *l* or *d* form, it may safely be concluded that compound formation has occurred.

The differences between the spectra of the racemic and optically active forms indicate in each of the observed cases except that of alanine that the racemic material is all or practically all

TABLE I
Wave Numbers and Intensities of Infra-Red Absorption Bands

Glycine		l-Alanine		dl-Alanine		l-Valine		dl-Valine		l-Leucine		dl-Leucine	
Wave No.	Intensity	Wave No.	Intensity	Wave No.	Intensity	Wave No.	Intensity	Wave No.	Intensity	Wave No.	Intensity	Wave No.	Intensity
cm. ⁻¹	per cent	cm. ⁻¹	per cent	cm. ⁻¹	per cent	cm. ⁻¹	per cent	cm. ⁻¹	per cent	cm. ⁻¹	per cent	cm. ⁻¹	per cent
2630	90	3030	90	2830	90	2980	90	2930	90	2930	90	2950	90
2045	10	2830	90	2680	90	2680	90	2580	90	2620	40	2560	90
1587	80	2640	10	2146	30	2123	50	2146	10	2137	20	2188	50
1508	90	2119	30	1597	90	1592	90	1605	90	1582	90	1597	90
1397	90	1597	90	1529	70	1520	90	1504	90	1508	80	1520	90
1316	80	1513	50	1462	40	1404	80	1416	60	1404	60	1416	70
1109	50	1453	50	1416	60	1335	70	1361	30	1353	50	1357	70
1028	40	1412	60	1361	70	1272	20	1319	70	1297	10	1302	60
912	70	1361	70	1309	70	1186	30	1267	20	1238	10	1236	30
894	60	1304	70	1241	30	1142	30	1181	20	1181	20	1181	10
699	70	1236	30	1153	20	1110	10	1132	30	1134	30	1136	40
609	30	1149	20	1114	60	1070	20	1104	10	1076	10	1083	10
527	50	1111	60	1015	40	1032	30	1066	10	1028	10	1036	20
503	60	1014	50	919	40	995	5	1033	30	998	5	996	20
		920	40	895	10	950	20	949	20	942	10	942	10
		894	5	851	80	925	20	925	20	916	20	923	30
		849	70	770	40	900	30	889	40	844	50	894	5
		772	40	583	5	888	30	820	30	832	20	849	60
		648	50	647	50	849	30	776	70	768	20	833	20
		583	5	546	70	826	10	686	60	704	10	775	30
		541	70	505	20	775	40	538	60	668	50	716	10
		506	20			750	10	476	40	649	5	685	70
		486	20			715	60	430	30	534	40	649	5
						664	30			516	10	581	10
						541	70			445	30	537	70
												521	20
												445	60

in the compound form. In the case of alanine the spectral differences are so slight that they do not preclude the existence of some *l* and *d* crystals in addition to the compound.

A curious result was obtained in the case of *dl*-glutamic acid monohydrate: This compound as prepared for infra-red observa-

TABLE II
Wave Numbers and Intensities of Infra-Red Absorption Bands

<i>l</i> -Phenyl- alanine		<i>dl</i> -Phenyl- alanine		<i>d</i> -Glutamic acid		<i>dl</i> -Glutamic acid		<i>l</i> -Tyrosine		<i>dl</i> -Aspartic acid		<i>dl</i> -Serine	
Wave No.	Intensity	Wave No.	Intensity	Wave No.	Intensity	Wave No.	Intensity	Wave No.	Intensity	Wave No.	Intensity	Wave No.	Intensity
cm. ⁻¹	per cent	cm. ⁻¹	per cent	cm. ⁻¹	per cent	cm. ⁻¹	per cent	cm. ⁻¹	per cent	cm. ⁻¹	per cent	cm. ⁻¹	per cent
3080	90	3030	90	2760	90	2980	90	2930	90	2990	90	2990	90
2450	90	2530	90	2137	10	1647	70	2114	10	2415	10	2430	10
2165	40	2203	40	1961	40	1515	90	1597	90	1961	10	2146	10
1562	90	1605	80	1695	60	1420	50	1529	10	1667	70	1642	70
1511	80	1504	80	1572	90	1353	70	1433	60	1597	70	1570	70
1412	60	1412	60	1462	70	1309	50	1370	50	1520	80	1504	70
1344	10	1342	10	1433	10	1244	80	1330	70	1416	50	1429	70
1312	70	1316	60	1377	20	1149	50	1248	70	1309	80	1353	60
1232	20	1211	20	1312	80	1124	70	1214	10	1250	70	1309	70
1160	40	1155	30	1276	90	1078	70	1160	30	1149	50	1244	30
1134	20	1073	20	1247	10	1056	70	1103	40	1119	50	1174	10
1076	30	1035	20	1166	60	970	20	1042	30	1080	10	1151	50
1029	30	987	10	1139	60	947	40	985	20	1044	50	1091	60
1002	10	948	5	1075	30	912	20	939	10	990	40	1032	80
951	10	912	20	1022	80	867	30	897	30	935	10	980	50
914	20	850	50	912	40	810	70	877	50	899	40	900	50
850	60	775	30	861	50	759	10	841	70	873	30	848	20
780	20	745	70	821	60	715	60	829	40	837	10	827	20
747	70	699	80	782	40	703	40	800	60	776	20	813	20
701	80	679	50	743	40	674	30	740	40	753	30	729	40
683	60	607	20	674	50	642	10	713	20	657	40	620	50
604	10	526	70	624	20	569	10	651	60	648	10	567	30
527	80	475	30	614	30	538	50	575	60	599	40	561	20
470	40			536	60	512	20	530	50	554	40	528	40
				514	50			494	20	461	30	502	40
				467	40			472	20				
				438	40								

tion possesses a spectrum identical to that of *l*-glutamic acid. A possible explanation is that powdering in absolute alcohol dehydrated the compound and left a mixture of the *d* and *l* crystals.

An interesting feature of the spectra of the amino acids is the behavior of the band due to the vibration of oxygen against carbon in the C=O group. In compounds containing the carboxyl group other than the amino acids this band generally occurs at a wavelength very near 5.9μ (wave number 1700 cm.^{-1}). It will be noticed, however, that in the monobasic monocarboxylic amino acids here studied this band regularly occurs at about 6.3μ (1590 cm.^{-1}). A plausible explanation for this shift is that a strong interaction exists between carboxyl and amino groups. This is substantiated by the spectra of the dicarboxylic acids, aspartic and glutamic acids, which have bands at about 6.0μ (1670 cm.^{-1}) in addition to the usual bands near 6.3μ . The bands occurring at 6.0μ very probably have their origin in the C=O of the second carboxyl group, which is not closely approached by an amino group.

SUMMARY

The infra-red absorption spectra of powder layers of nine different amino acids have been recorded in the range from 3 to 24μ .

In the case of five of these acids both the racemic form and an optically active form have been observed. Differences of these spectra have shown that in each case the racemic form, as crystallized from solution, is a compound, not a mixture of *l* and *d* crystals.

Shifts in the position of the C=O band have indicated a strong interaction between carboxyl and amino groups in the crystalline amino acids.

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STUDIES ON BIOLOGICAL OXIDATIONS

XI. THE METABOLISM OF PYRUVIC ACID BY ANIMAL TISSUES AND BACTERIA

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Pyruvic acid is one of the most reactive substances among those of importance in cellular metabolism. When carbohydrate is split during fermentation, pyruvic acid is the main end-product of the resulting series of phosphorylated oxidation-reductions. It thus becomes the immediate source for the formation of both lactic acid and alcohol. Pyruvic acid is decarboxylated by yeast; it may react with other ketonic acids in a Cannizzaro type reaction (dismutation) (Krebs (1)); it may likewise react with amino acids with intermolecular transfer of amino groups (Braunshtein and Kritsman (2)), the orientation of pyruvic acid metabolism being determined by a variety of different factors.

The oxidation of pyruvic acid by gonococci was studied in 1932 by Barron and Miller (3) who postulated the existence of an enzyme, α -ketonoxidase, able to catalyze the oxidation of α -ketonic acids. Other properties of this enzyme were later studied by Barron (4). To distinguish it from α -hydroxyoxidase, the enzyme concerned with the oxidation of lactic acid, a number of inhibitors were found having opposite effects on the two enzymes. Krebs and his coworkers (5, 6) challenged this interpretation and postulated that the oxidation of α -ketonic acids is not brought about by molecular oxygen, but by a dismutation. In this paper an endeavor is made to show that pyruvic acid in the presence of oxygen is oxidized directly; that the dismutation of pyruvic acid observed by a number of authors and confirmed by Krebs and his coworkers is a process independent of the oxidation process. Furthermore, Lipmann's studies (7) on the action

of diphosphothiamine as a catalyst for the oxidation of pyruvic acid have been confirmed by experiments with a variety of bacterial cells and animal tissues. Diphosphothiamine was also found to act as a catalyst for the dismutation of pyruvic acid.

EXPERIMENTAL

The bacteria were grown in flat medicine bottles: hemolytic streptococci on a beef infusion-blood agar medium without glucose, gonococci in Miller and Castles' egg digest medium containing 1 per cent glucose (8), and staphylococci in a medium containing 1 per cent proteose-peptone, 0.3 per cent meat extract, 0.5 per cent NaCl, and 3 per cent agar, all dissolved in tap water. The bacteria were washed twice with 0.154 M NaCl; after centrifugation they were suspended in the buffer solutions used for the experiments. The O₂ uptake and CO₂ output were measured with the usual Warburg-Barcroft manometers. The pH values of the suspensions were measured with the glass electrode. Pyruvic acid was determined manometrically by oxidation with ceric sulfate and measurement of the CO₂ output (in strong acid solutions (0.1 M H₂SO₄) pyruvic acid is entirely oxidized within 7 minutes). Lactic acid was determined manometrically by oxidation with α -hydroxyoxidase obtained from a suspension of gonococci in 0.1 M NaF. (Under these conditions lactic acid is oxidized to pyruvic acid: $\text{CH}_3\text{CHOHCOOH} + \frac{1}{2}\text{O}_2 = \text{CH}_3\text{COCOOH} + \text{H}_2\text{O}$.) Acetic and formic acids were determined by adapting Friedemann's method (9) to the measurement of small quantities of volatile acids. Acetic acid was identified by Krüger and Tschirch's (10) lanthanum reaction. Thiamine (synthetic) was obtained through the courtesy of Merck and Company, Inc. Diphosphothiamine was prepared by the method of Lohmann and Schuster (11) from fresh beer yeast, obtained through the courtesy of the Keeley Brewing Company of Chicago. Diphosphothiamine can be differentiated from thiamine with Prebluda and McCollum's diazo reagent (12). With this reagent, thiamine gives a red-purple color, extractable with isobutyl alcohol. Diphosphothiamine gives a yellowish brown color when relatively high concentrations are used. At lower concentrations, at which thiamine gives quite a distinct color (10 to 20 micrograms per cc. of solution to be tested), diphosphothiamine gives no visible color.

The experiments in which O_2 uptake was measured were performed in phosphate buffers with either air or oxygen as gas phase; those in which CO_2 output was measured were performed in bicarbonate buffer with CO_2-N_2 as gas phase. The N_2-CO_2 gas mixtures were freed from traces of oxygen by being passed through a 1 meter Pyrex tube filled with copper pellets and heated to 500° .

The preparation of the yeast enzyme for the detection and estimation of cocarboxylase was essentially that of Lohmann and Schuster (11) with the following modification. Preliminary to washing the dried yeast with a disodium phosphate solution, it was allowed to stand in distilled water for 20 minutes and then centrifuged. The yeast was then washed twice with 0.1 M Na_2HPO_4 (50 cc. per gm. of dried yeast) at 30° , the two washings being completed in a total of 12 minutes, including the necessary time for centrifugation. After washing again with distilled water, the yeast was suspended in M/15 phosphate, pH 6.2. The shortened period of washing with the basic sodium phosphate solution was made possible by the preliminary treatment with distilled water. The above procedure gave an enzyme preparation which was very active for the decarboxylation of pyruvic acid in the presence of added cocarboxylase, either natural or synthetic. Without the addition of cocarboxylase the enzyme was always almost completely devoid of activity.

Oxidation and Dismutation of Pyruvic Acid by Bacteria

Gonococci—Barron and Miller (3) found that these bacteria possess only two oxidizing enzymes: α -hydroxyoxidase, which catalyzes the oxidation of α -hydroxy acids to α -ketonic acids, and α -ketonoxidase, which catalyzes the oxidation of pyruvic acid to acetic acid and CO_2 . The first reaction is thermodynamically reversible (Barron and Hastings (13)); the second is irreversible (Barron (4)). *In the presence of oxygen* both oxidative reactions go to completion. Krebs (5) found that *in the absence of oxygen* pyruvic acid was dismutated by gonococci according to the following equation.



We have confirmed Krebs' experiments. Because of this anaerobic dismutation, Krebs (5) concluded, "Pyruvic acid is oxidized in

Gonococcus by the anaerobic reaction (1), and the oxygen uptake which ensues after addition of pyruvic acid is due to a secondary oxidation of the lactic acid formed by reaction (1)."

If the oxidation of pyruvic acid by gonococci proceeds according to Krebs' scheme, the following conditions must be fulfilled: (1) The rate of CO_2 output in the absence of oxygen (optimum conditions for dismutation) must be greater than the rate of O_2 uptake in the presence of oxygen; (2) whenever the oxygen uptake in the presence of pyruvic acid is inhibited, this must be due to inhibition either of the anaerobic reaction (Equation 1) or of the oxidation

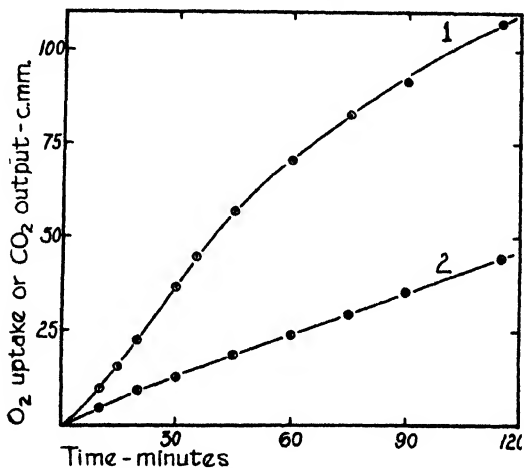


FIG. 1. Metabolism of pyruvic acid by gonococci. pH 7.02, temperature 38° , amount of lithium pyruvate 0.01 mM. Curve 1, in air, oxidation as measured by the O_2 uptake. Curve 2, in $\text{N}_2\text{-CO}_2$, dismutation as measured by the CO_2 output.

of the lactic acid produced in that reaction; (3) any substance which inhibits the oxidation of lactic acid must also inhibit pyruvic acid oxidation.

The rate of O_2 uptake by gonococci in the presence of pyruvate (oxidation) was greater than the rate of CO_2 output in the absence of oxygen (dismutation), showing that condition (1) was not fulfilled (Fig. 1). (Krebs did not measure the rates of CO_2 production and O_2 uptake during the metabolism of pyruvic acid by gonococci.)

NaF has no effect on the oxidation of lactate to pyruvate by

gonococci; it inhibits considerably the oxygen uptake of gonococci in the presence of pyruvate (Barron (4)). If the metabolism of pyruvic acid by gonococci, *in the presence of oxygen*, is performed according to Krebs' scheme, this inhibiting effect of NaF must then be due to inhibition of dismutation. In the absence of oxygen, the rate of CO₂ output by gonococci with pyruvate was not inhibited by 0.02 M NaF. Indeed, the rate of CO₂ output was slightly greater in the presence of NaF (Fig. 2). Condition (2) was therefore not fulfilled.

H₂S inhibits the oxidation of lactic acid by gonococci almost completely; it must therefore inhibit the O₂ uptake in the presence of pyruvate if Krebs' scheme is correct. The O₂ uptake and CO₂ output were measured simultaneously by Warburg's indirect method (14). The bacteria were suspended in 0.2 M KH₂PO₄, and the O₂ uptake and CO₂ output were measured in the presence of 0.0015 M H₂S at 28°. In 28 minutes the O₂ uptake with pyruvate was 93 c.mm. and the CO₂ output, 140 c.mm. The O₂ uptake with lactate was 11 c.mm. The O₂ uptake in these experiments with pyruvate as substrate cannot then be due to the oxidation of lactic acid formed by dismutation of pyruvic acid, since lactate oxidation was considerably suppressed. Obviously, condition (3) was not fulfilled.

Streptococcus hemolyticus—Barron and Jacobs (15) found one strain of *Streptococcus hemolyticus* which oxidized pyruvic acid; neither lactic, succinic, acetic, nor formic acid was oxidized. In the presence of air, pyruvic acid took up 1 atom of oxygen and produced 1 mole of CO₂ per mole of pyruvate. On chemical analysis, there was found 0.01 mM of acetic acid (identified by Krüger and Tschirch's lanthanum reaction) per 0.01 mM of pyruvate oxidized.

In the absence of oxygen, pyruvic acid was converted into acetic acid and formic acid.



The CO₂ output in bicarbonate buffer with CO₂-N₂ as the gas phase was from 98 to 72 per cent of the theoretical value, calculated according to Equation 2. Six experiments were done on different days (Table I). In experiments with phosphate buffer and N₂ as the gas phase there was an output of 17 c.mm. of CO₂ per 0.01 mM of pyruvate in 180 minutes at 38°. (The origin of

this CO_2 output was not determined.) No hydrogen was given off. For the determination of volatile acids, the bacterial suspension containing pyruvate with N_2 as gas phase was kept rotating for 3 hours at 38° ; acetic and formic acids were found (Table I). Since neither acetic nor formic acid is oxidized by this strain of hemolytic streptococci, it is obvious that in these cells the metabolism of pyruvic acid in the presence and the absence of oxygen

TABLE I

Anaerobic Metabolism of Pyruvic Acid by Hemolytic Streptococci
Analysis of End-Products of Reaction

Phosphate buffer, pH 7.02 (initial); N_2 as gas phase; temperature 38° .

Acid	Before incubation	After incubation
	mm	mm
Pyruvic.....	0.020	0.003
Acetic.....	None	0.017
Formic.....	"	0.014

Extent of Reaction As Measured by CO_2 Liberation from Bicarbonate Buffer
 pH 7.02 (initial); gas phase, $\text{N}_2\text{-CO}_2$; temperature 38° ; calculated CO_2 output: 1 mole of CO_2 per mole of pyruvic acid ($\text{CH}_3\text{COCOOH} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COOH} + \text{HCOOH}$).

Experiment No.	CO_2 output		Extent of reaction
	Found	Calculated	
	c.mm.	c.mm.	per cent
1	317	440	72
2	368	418	88
3	208	212	98
4	330	440	75
5	370	421	88
6	351	398	88

follows different paths—the first giving acetic acid and CO_2 as end-products, the second giving acetic acid and formic acid as end-products.

Staphylococci—In his paper on bacterial metabolism of pyruvic acid, Krebs found that *Staphylococcus aureus* oxidized pyruvate to about one-sixth of complete oxidation. (The bacteria were grown on broth agar.) Sevag and Neuenschwander-Lemmer (16)

had previously reported that bouillon cultures of *Staphylococcus aureus* did not oxidize pyruvic acid. Five different strains of *Staphylococcus aureus*, isolated by Dr. T. E. Friedemann and kindly given to us by him, all oxidized pyruvic acid further than one-sixth of complete oxidation; one of them oxidized it to completion and the other four oxidized it readily to three-fifths of completion. All of these bacteria oxidized formic acid readily,

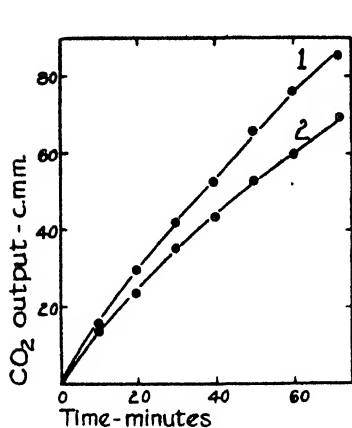


FIG. 2

FIG. 2. Dismutation of pyruvic acid by gonococci. pH 7.02, temperature 38°, gas phase N_2 - CO_2 . CO_2 output: Curve 1, in the presence of NaF (0.02 M); Curve 2, control.

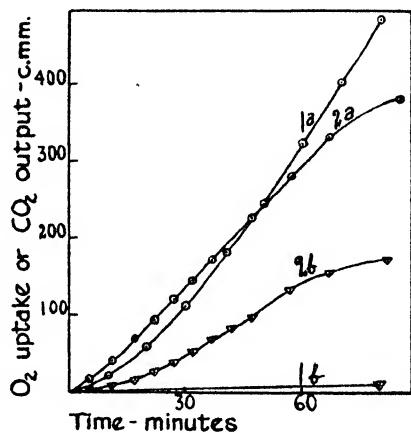


FIG. 3

FIG. 3. Metabolism of pyruvic acid by staphylococci in the presence and absence of oxygen, as measured by the O_2 uptake in phosphate buffer with air as the gas phase, and the CO_2 output in bicarbonate buffer with N_2 - CO_2 as the gas phase. pH 7.02, temperature 38°. In each case a represents O_2 uptake; b, CO_2 output. Curve 1 represents *Staphylococcus aureus*; Curve 2, *Staphylococcus albus*.

the rate of oxidation of acetic acid varying in the different strains. Of three strains of *Staphylococcus albus* studied, two oxidized pyruvate to completion and one to three-fifths of complete oxidation. It may be mentioned that all those strains of staphylococci which did not oxidize pyruvic acid completely showed a low rate of acetate oxidation (Table II).

In the absence of oxygen there was pyruvic acid dismutation according to Krebs' scheme. The rate of dismutation by the strain

of *Staphylococcus aureus* studied by Krebs (as measured by the CO₂ output in bicarbonate with N₂-CO₂ as gas phase) was greater than the rate of oxidation (as measured by the O₂ uptake in the presence

TABLE II

Oxidation of Pyruvic, Acetic, and Formic Acids by Staphylococci

Phosphate buffer, pH 6.99; temperature 38°; amount of pyruvate, acetate, and formate, 0.01 mm.

Strain	O ₂ uptake per hr.		
	Pyruvate	Acetate	Formate
	c.mm.	c.mm.	c.mm.
<i>Staphylococcus albus</i> (No. A44)	405	200	110
“ <i>aureus</i> (variant of No. A44)	382	122	116
“ <i>albus</i>	218	96	120
“ “ (8 yrs. old)	106	22	61
“ <i>aureus</i> (hemolytic, freshly isolated)	320	23	106
“ “ “ “ “ “	278	22	120
“ “ (8 yrs. old)	65	6	70
“ “ (acetone-treated)	33	0	113

TABLE III

Oxidation of Lactic, Acetic, and Formic Acids by Acetone-Treated Staphylococcus aureus

Temperature 38°; pH 6.99; amount of oxidizable substrates, 0.01 mm.

Time	O ₂ uptake		
	Lactate	Pyruvate	Formate
	c.mm.	c.mm.	c.mm.
min.			
6	16.9	3.2	16.2
10	26.9	6.0	27.7
20	45.1	10.3	61.0
30	57.8	17.0	85.1
40	62.4	21.1	100.0
50	69.2	28.9	109.7
60	72.4	33.7	114.6

of air). He considered this difference in rates an indication in favor of his theory, already referred to. In none of the eight different strains of staphylococci reported here was the CO₂ output greater than the O₂ uptake. In Fig. 3 two extreme cases are

reported: in one case (*Staphylococcus albus*) there was a vigorous CO_2 output, although not as great as the O_2 uptake; in the other case (*Staphylococcus aureus*), the rate of CO_2 output was negligible compared to the rate of O_2 uptake.

This great difference in the rates of oxidation and dismutation with *Staphylococcus aureus* makes it possible to separate the oxidation of pyruvic acid from the dismutation process. The bacteria were washed three times with water; after centrifugation they were frozen with a mixture of dry ice and ether, precipitated with acetone, and again centrifuged; the precipitate was washed with ice-cold acetone and collected in a Gooch filter, where it was washed again with ice-cold acetone and ether. This acetone-treated powder oxidized lactic, pyruvic, and formic acids. It had lost the power to produce either dismutation of pyruvic acid or oxidation of acetic acid (Table III).

Oxidation and Dismutation of Pyruvic Acid by Animal Tissues

Krebs and Johnson (6) have elaborated a theory on the mechanism of the metabolism of ketonic acids, which is similar to the mechanism proposed by Krebs for bacteria.

The reduction of pyruvic acid to lactic acid by animal tissues has been reported by numerous investigators (Mayer (17), Elliot *et al.* (18), Lawson (19)). Pyruvic acid may also give rise to the formation of succinic acid (Weil-Malherbe (20), Elliot and Greig (21), Krebs and Johnson (6)), acetic acid (Weil-Malherbe (20), Krebs and Johnson (6)), acetoacetic acid (Embden and Oppenheimer (22)), and β -hydroxybutyric acid (Krebs and Johnson (6)).

If the metabolism of pyruvic acid in animal tissues follows the same initial path in the presence and in the absence of air, then the rate of consumption of pyruvic acid in bicarbonate buffer with $\text{N}_2\text{-CO}_2$ as gas phase would not be expected to be less than in the presence of air. McGowan (23) and McGowan and Peters (24), in their work on the oxidation of pyruvic acid by avitaminotic brain tissue, and Weil-Malherbe (25), in his work on the anaerobic dismutation of pyruvic acid by rat brain tissue, have offered evidence against Krebs' theory. In Fig. 4 the rates are given for the CO_2 output (in Krebs' bicarbonate solution with $\text{CO}_2\text{-N}_2$ as gas phase) and the O_2 uptake (in Krebs' phosphate solution with O_2 as gas phase) of the following tissues: liver, kidney, and brain

(from avitaminotic pigeons); brain and testis (rat); and nucleated erythrocytes (goose). Ground brain cortex was previously washed with cold distilled water, as done by Peters; liver and kidney were used in thin slices; the goose erythrocytes were washed three times with 0.154 M NaCl. The figures given were obtained

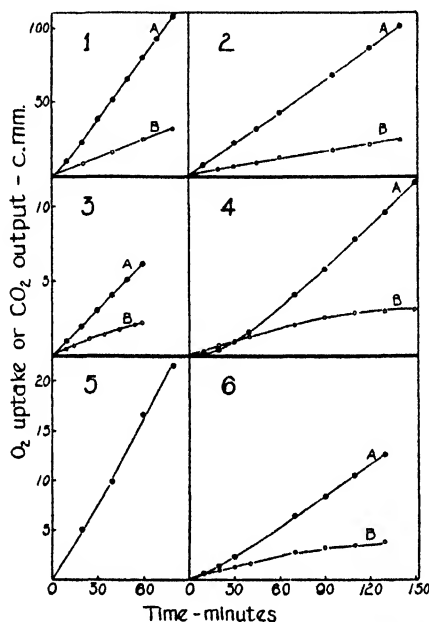


FIG. 4. Metabolism of pyruvic acid by animal tissues. Curves A represent the O₂ uptake with oxygen as the gas phase, in Krebs' phosphate solution; Curves B, the CO₂ output with N₂-CO₂ as the gas phase, in Krebs' bicarbonate solution. 1 represents normal rat brain, per 100 mg. of moist tissue; 2, avitaminotic pigeon brain with thiamine added, per 100 mg. of moist tissue; 3, normal rat testes, per mg. of dry weight; 4, avitaminotic pigeon kidney with thiamine added, per mg. of dry weight; 5, goose erythrocytes; 6, avitaminotic pigeon liver with thiamine added, per mg. of dry weight.

by subtracting the gas uptake or output in the absence of pyruvate. In no case was the rate of CO₂ output greater than or even equal to the rate of O₂ uptake. The goose erythrocytes oxidized pyruvic acid but could not utilize it in the absence of oxygen. Furthermore, while in every case the rate of O₂ uptake increased, up to a

certain time, to remain as a zero order reaction afterwards, the rate of CO_2 output in the absence of oxygen decreased with time in all tissues.

Oxydismutation Coefficient of Pyruvic Acid

The oxydismutation coefficient of pyruvic acid, *i.e.* the ratio between the amount of pyruvic acid used by the cell under conditions optimum for oxidation and the amount used under con-

TABLE IV

Oxydismutation Coefficient of Pyruvic Acid by Bacteria

Temperature 38°; pH 7.02. Oxidation, in phosphate buffer (air as gas phase); dismutation, in bicarbonate buffer ($\text{CO}_2\text{-N}_2$ as gas phase).

Bacteria	Oxidation	Dismutation	Oxydismutation coefficient, pyruvate used in air pyruvate used in N_2
	per cent	per cent	
Gonococci	33.0	10.7	3.1
<i>Streptococcus hemolyticus</i>	47.8	58.1	0.8
<i>Staphylococcus aureus</i>	35.3	30.4	1.16
“ “ (No. A43)	34.1	3.6	9.5
“ “ (“ A9, 8 yrs. old)	9.8	0.98	10.0
“ “ (hemolytic)	47.6	3.8	12.5
“ “ (freshly isolated)	67.6	4.5	15.0
“ <i>albus</i>	26.4	61.2	0.43
“ “	19.5	17.9	1.1
“ “ (variant of No. A43) ..	36.2	19.2	1.9
“ “ (No. A4, 8 yrs. old) ..	15.7	0	∞

ditions optimum for dismutation, was determined for bacteria after incubation at 38° for 1 hour. Only in the cases of *Streptococcus hemolyticus* and one strain of *Staphylococcus albus* was the rate of pyruvic acid disappearance greater in the absence of oxygen than in the presence of oxygen. One strain of *Staphylococcus albus* did not split pyruvic acid anaerobically. The others had an oxydismutation coefficient greater than 1 (Table IV).

In animal tissues (rat) also the oxydismutation coefficient is

greater than 1, as calculated from data given by Krebs and Johnson in Table II of their paper (6). From Krebs and Johnson's data the oxydismutation coefficient in rat brain is 1.32. Weil-Malherbe (25) maintains that the anaerobic metabolism of pyruvic acid in rat brain slices is 3 times as low as the aerobic metabolism. In this case the oxydismutation coefficient would be 3. Retina produces little or no CO_2 with pyruvic acid in the absence of oxygen, while it shows a large O_2 uptake in the presence of oxygen (Passenti (26)).

Diphosphothiamine, a Catalyst for Oxidation and Dismutation of Pyruvic Acid

Peters (27) discovered that thiamine (vitamin B_1) increased the rate of oxidation of pyruvic acid by brain tissue from avitaminotic pigeons. Shortly after the chemical constitution of cocarboxylase (diphosphothiamine) was determined by Lohmann and Schuster (11), Lipmann (7) found that this substance was also necessary for the oxidation of pyruvic acid by acetone-treated *Bacterium delbrückii*. Although Peters (28) was unable to confirm Lohmann and Schuster's contention that diphosphothiamine increases the rate of oxidation of pyruvic acid by brain tissue of avitaminotic pigeons as much as thiamine, the fact that thiamine is rapidly phosphorylated by animal tissues and yeast (Tauber (29), von Euler and Vestin (30), Lohmann and Schuster (11), Peters (28), Lipschitz, Potter, and Elvehjem (31)) favors the opinion that the catalyst for the oxidation of pyruvic acid is diphosphothiamine. This phosphorylating power of animal tissues makes them unsuitable for deciding whether thiamine has to be phosphorylated before it becomes a catalyst for the oxidation of pyruvic acid.

Since the results of Peters concerning the effect of diphosphothiamine and thiamine did not agree with those of Lohmann and Schuster, we repeated these experiments using brain from avitaminotic pigeons to oxidize pyruvic acid in the absence and in the presence of thiamine, yeast diphosphothiamine, and synthetic diphosphothiamine (kindly sent to us by Dr. H. Tauber). The brain tissue was minced and washed once with ice-cold saline. At the end of 90 minutes there was an increase of 97 per cent with thiamine and 33 per cent with both natural and synthetic diphosphothiamine (Fig. 5).

In order to determine whether thiamine must be phosphorylated to act as a catalyst for pyruvic acid oxidation, a cell should be chosen that is unable to phosphorylate thiamine but able to oxidize pyruvic acid. Such conditions were found in gonococci and hemolytic streptococci. (All bacterial suspensions used were washed several times with distilled water before the experiments.) Addition of thiamine to these bacteria (10 micrograms for 2.7 cc.

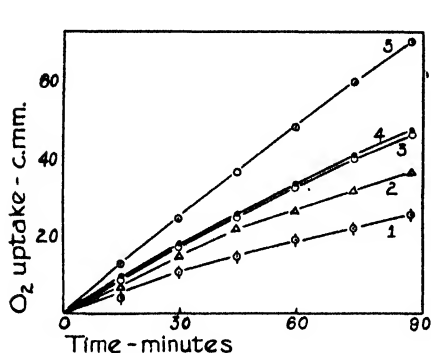


FIG. 5

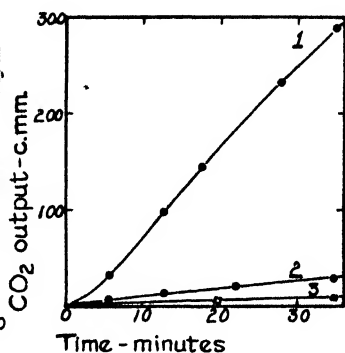


FIG. 6

FIG. 5. Effect of thiamine and of natural and synthetic diphosphothiamine on the oxidation of pyruvate by brain cortex (avitaminotic pigeon). Amount of pyruvate 0.03 mm, pH 7.4, temperature 38°. Curve 1 represents absence of pyruvate; Curve 2, presence of pyruvate, without thiamine; Curves 3 and 4, effect of added natural and synthetic diphosphothiamine (14 micrograms); Curve 5, effect of added thiamine (10 micrograms).

FIG. 6. Cocarboxylase activity of *Staphylococcus aureus* grown in a synthetic medium containing 4×10^{-5} M thiamine. Carboxylase was prepared from alkaline washed yeast. Temperature 28°, pH 6.2, amount of lithium pyruvate 5.4 mg. per vessel. Curve 1 represents the fluid from boiled bacteria (0.7 cc.); Curve 2, the supernatant fluid from the culture medium (2 cc.); Curve 3, enzyme plus pyruvate.

of bacterial suspension added 3 hours before the addition of pyruvate and kept at 38°) had no influence on the rate of O₂ uptake; addition of diphosphothiamine (14 micrograms added 30 minutes before addition of pyruvate) increased the O₂ uptake by 21 per cent in gonococci, and 64 per cent in hemolytic streptococci. Diphosphothiamine had very little effect (8 per cent increase) on the oxidation of pyruvate by *Staphylococcus aureus* (Table V).

TABLE V

Effect of Thiamine and Diphosphothiamine on Oxidation and Dismutation of Pyruvic Acid by Animal Tissues and Bacteria

Amount of lithium pyruvate, 0.01 mM; thiamine, 10 micrograms; diphosphothiamine, 14 micrograms.

Bacteria	Catalyst	Oxidation			Dismutation		
		O ₂ uptake per hr.		Increase	CO ₂ output per hr.		Increase
		Control	With catalyst		Control	With catalyst	
		c.mm.	c.mm.	per cent	c.mm.	c.mm.	per cent
Gonococci.....	Diphosphothiamine	43.0	52.0	21.0	24	27.0	12.5
".....	Thiamine	57.0	56.0	None			
<i>Streptococcus hemolyticus</i> ..	Diphosphothiamine	82.0	135.0	64.6	67	80.0	19.4
" ".....	Thiamine	34.7	33.8	None			
<i>Staphylococcus aureus</i>	Diphosphothiamine	470.3	507.9	8.0			
" ".....	Thiamine	470.3	473.0	None			
Same, washed twice with 0.1 M Na ₂ HPO ₄	Diphosphothiamine	124.0	171.0	37.6	0	23.0	
" ".....	Thiamine	195.0	220.0	12.8			
Erythrocytes, goose.....	"	14.0	17.0	21.4	0		
" ".....	Diphosphothiamine	14.0	16.5	18.0			
Brain (avitaminotic pigeon).....	Thiamine	26.5	48.5	80.0	12.2	17.6	44.0

When the last mentioned bacteria were shaken twice for 10 minutes with 0.1 M Na₂HPO₄ at 30°, centrifuged, and washed with water, the rate of pyruvate oxidation increased by 38 per cent

on addition of diphosphothiamine; addition of thiamine 2 hours before pyruvate addition produced an increase of 13 per cent. This may be taken as an indication that staphylococci have the power of phosphorylating thiamine. (After these experiments were performed there appeared Hills' recent report (32) that the rate of pyruvate oxidation by staphylococci grown in a synthetic medium containing minimal amounts of thiamine increased on addition of thiamine.)

The power of staphylococcus to phosphorylate thiamine has been demonstrated as follows: *Staphylococcus aureus* was grown in the synthetic medium recommended by Fildes and coworkers (33), the thiamine content being increased to 4×10^{-5} M. After

TABLE VI

Effect of Inhibitors on Decarboxylation (by Yeast) and Oxidation (by Gonococci) of Pyruvic Acid

Inhibitor	Inhibition	
	Decarboxylation	Oxidation
	per cent	per cent
α -Naphthol, 0.001 M.	19.6	93
HCN, 0.01 M.	6.3	
" 0.002 "	None	77
NaF, 0.02 "	"	
" 0.01 "	"	75

incubation at 38° for 16 hours, the bacteria from 150 cc. of the culture were collected by centrifuging. 3 cc. of water were added to the bacteria, and the suspension was boiled for 5 minutes, cooled, and centrifuged. 0.7 cc. of the supernatant fluid was used in the test experiments for cocarboxylase activity. This fluid from boiled bacteria, together with yeast which had been freed from cocarboxylase by washing with 0.1 M disodium phosphate, decarboxylated pyruvic acid vigorously (Fig. 6). The supernatant fluid from the culture (2 cc.), on the other hand, had very little activity. Thiamine phosphorylated by the bacteria was therefore mostly retained within the cell.

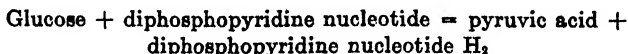
Diphosphothiamine was also found to accelerate the rate of CO₂ output in anaerobic experiments performed with gonococci,

hemolytic streptococci, washed staphylococci, and brain tissue from avitaminotic pigeons (Table V).

Decarboxylation and Oxidation of Pyruvic Acid—In a recent paper, Cedrangolo (34) maintains that the oxidation of pyruvic acid by ground liver is performed by previous decarboxylation to acetaldehyde, which is later oxidized to acetic acid. As proof for this contention he presents experiments in which the oxygen uptake of ground liver was inhibited by 80 per cent on addition of dimethyl cyclohexanedione. If pyruvic acid is split via acetaldehyde, those substances which act as powerful inhibitors of pyruvic acid oxidation may be expected also to inhibit its decarboxylation. NaF, α -naphthol, and HCN are strong inhibitors for the oxidation of pyruvic acid (Barron (4)). Neither fluoride nor HCN inhibited decarboxylation. α -Naphthol, which inhibits completely the oxidation of pyruvic acid, inhibited by 20 per cent its decarboxylation. In Table VI are given the values for the inhibiting effect of these substances on the decarboxylation of pyruvic acid by dried yeast and on its oxidation by gonococci. We may conclude from these experiments that decarboxylation and oxidation of pyruvic acid are independent processes.

DISCUSSION

The rôle of pyruvic acid in the oxidation-reduction reactions which occur in living cells is becoming more important as the mechanism of intermediary metabolism is disentangled. In the presence of the activating enzyme, pyruvic acid becomes extremely reactive and will react in a variety of different ways, the end-products depending on the presence of other reacting substances and the oxidation-reduction intensity and capacity of the milieu. Thus, during the process of carbohydrate fermentation (in glycolysis and likewise in alcoholic fermentation), the splitting of the glucose molecule results in the formation of pyruvic acid. This is accomplished through a series of oxidation-reduction reactions performed with the mediation of diphosphopyridine nucleotide.



The resulting pyruvic acid may be reduced to lactic acid (glycolysis); it may be decarboxylated and end in alcohol (alcoholic

fermentation); it may react with amino acids, ketonic acids, or ketones, acting either as a reductant or as an oxidant in quite a number of coupled oxidations. The direction and the extent of these reactions will be determined by the value of the constants of the equilibrium reactions taking place under the conditions of the experiment. Disregard of these premises has led numerous investigators to erroneous conclusions concerning the mechanism of carbohydrate metabolism.

The experiments reported in this paper show that the orientation of pyruvic acid metabolism depends also on the oxygen tension of the milieu. In the simplest systems among those studied, that is, in cells containing no α -hydroxyoxidase (the enzyme concerned with the reversible oxidation-reduction, $\text{pyruvate} + 2e + 2\text{H}^+ \rightleftharpoons \text{lactate}$), for example, hemolytic streptococci, pyruvic acid was either oxidized to acetic acid and CO_2 or split into acetic and formic acids. The first process was found to go to completion in the presence of oxygen at atmospheric tension; the second went to completion in the absence of oxygen. In more complicated systems, cells possessing α -hydroxyoxidase (gonococci), pyruvic acid was oxidized in the presence of oxygen, while in its absence it was partly reduced to lactic acid and partly oxidized to acetic acid and CO_2 . It is obvious that the relation between oxidation and dismutation, oxydismutation coefficient, will vary in each cell according to the oxygen tension.

Both the oxidation and dismutation of pyruvic acid require diphosphothiamine as one of the factors of the enzyme complex, the phosphorylation of thiamine being easily performed by animal tissues (29, 30, 11, 28, 31) and by bacteria. The contention of Cedrangolo (34) that the first product of pyruvic acid metabolism is acetaldehyde (decarboxylation) finds no support in our inhibition experiments, because inhibitors of pyruvic acid oxidation had no effect on the rate of decarboxylation.

SUMMARY

The metabolism of pyruvic acid by bacteria (gonococci, hemolytic streptococci, staphylococci) and animal tissues (pigeon, rat) may proceed in two directions: Under optimum conditions for oxidation, the pyruvic acid is directly oxidized to acetic acid and CO_2 ; under optimum conditions for reduction it may be reduced

to lactic acid or may be split by dismutation into acetic acid and formic acid. By keeping bacteria under these optimum conditions, the oxydismutation coefficient of pyruvic acid was determined for gonococci, hemolytic streptococci, and staphylococci. Goose erythrocytes oxidize pyruvic acid in air. In the absence of oxygen, pyruvic acid is not utilized. Diphosphothiamine acts as a catalyst for both the oxidation and the dismutation of pyruvic acid.

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THE PHYLLOCHROMOGEN OF PROTOPORPHYRIN AND PYRIDINE

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The absence of photosynthetic activity in all plant tissues in which the cell structure has been destroyed, as well as in chlorophyll extracts from plants, would indicate a destruction of the natural photosynthetic system by these processes. No labile or sensitive forms of chlorophyll have been obtained; it would seem, therefore, that the pigment portion is unchanged, but that the disruption of its association with non-color-absorbing matter in the chloroplast accounts for the absence of photosynthesis.

Analogy between the heme-globin system of hemoglobin and the chlorophyll synthetic system suggests that chlorophyll is associated with protein or other nitrogen-containing material. This idea is not novel; nor is there complete absence of support for it. Willstätter and Stoll (1) observed a shift towards the violet in the absorption spectrum of chlorophyll after extraction. This was attributed to the passage of chlorophyll from a colloidal state to one of true solution. Such a shift occurs when the mass of non-absorbing matter associated with a chromophoric group is diminished; it would occur if chlorophyll were dissociated from a protein, as has been suggested by Lubimenko (2). The latter was able to obtain aqueous extracts of chlorophyll from *Aspidistra* and *Funkia* in which the absorption was not changed from that in the leaf. He also found (3) that protein reagents such as tannic acid or ammonium sulfate precipitated the pigment from solution, and that the complex was broken up by organic solvents, giving a green solution and colorless residue. This behavior supports the theory that chlorophyll is present in the chloroplast as the prosthetic group of a protein complex compound.

* Contribution No. 176.

Preliminary to a study of the affinity of chlorophyll for protein, the behavior of the inner magnesium salts of porphyrins, the phyllins, with simple nitrogen compounds has been investigated. Such phyllins have been found to form stable complex salts with pyridine, like the hemochromogens from heme and nitrogen compounds. They are accordingly named phyllochromogens.

The pyridine phyllochromogen of protoporphyrin methyl ester may be prepared by recrystallizing the phyllin ester from ether containing pyridine. It separates as a microcrystalline powder which may be filtered off, washed with petroleum ether, and dried by exposure to the air. Exactly 2 moles of pyridine are given up when protophyllochromogen is warmed in a vacuum; the resulting phyllin will also recombine with 2 moles of pyridine when it is allowed to stand in an atmosphere saturated with pyridine. The product is therefore a bipyridine compound, as are the pyridine hemochromogens (4, 5).

The arrangement of the pyridine groups may be assumed to be like that in hemochromogens, the magnesium atom occupying the center position within the porphyrin nucleus and the pyridine groups the positions above and below this plane. Magnesium forms complex salts with valencies of both 4 and 6, the former being the more stable (6). Of those with 6 covalencies, besides alcohols, esters, and ketones within the complex, there are those with amides ($\text{Mg}(\text{CH}_3\text{CONH}_2)_6\text{Br}_2$), urea ($\text{Mg}(\text{CON}_2\text{H}_4)_6\text{I}_2$), and urethanes ($\text{Mg}(\text{NH}_2\text{CO}_2\text{C}_2\text{H}_5)_6\text{Br}_2$) (7).

Protophyllin ester gives in ether a cherry-red solution, and in pyridine a red solution with a pronounced yellow-green tinge. In pyridine the absorption spectrum shows a shift towards the red of $7\text{ m}\mu$ for Band I and of $10\text{ m}\mu$ for Band II. Fig. 1 shows the curves from the Hardy color analyzer.¹ In dilute solutions of pyridine in ether, the extent of phyllochromogen formation is dependent upon the concentration of pyridine, as shown by the absorption. When green leaves are treated with acetone, the strong red band of chlorophyll moves $12\text{ m}\mu$ to the blue (1); a similar shift occurs when pyridine phyllochromogen is broken up by dilution.

Phyllins may therefore combine with nitrogen compounds in

¹ The author wishes to thank Mr. S. Q. Duntley of the Massachusetts Institute of Technology for his cooperation in making these curves.

the same manner as do the heme derivatives of porphyrins. The dissociation of the resulting complex is accompanied by spectral shifts analogous to those occurring when chlorophyll is extracted from plant tissues. These characteristics lend support to the occurrence of chlorophyll in nature as a nitrogen complex salt. It is hoped that the behavior of phyllins and chlorophyll with

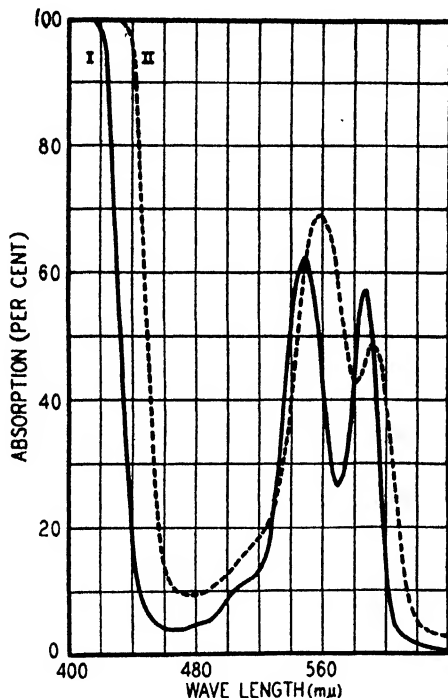


FIG. 1. The absorption spectra of protophyllin dimethyl ester in ether (Curve I), and in pyridine (Curve II).

proteins, which is now being studied, will throw further light on this association.

It is significant that French (8) has recently obtained from photosynthetic bacteria a complex which appears to consist of protein and chlorophyll and carotenoid pigments. Smith (9) has also reported the preparation of an aqueous chlorophyll protein solution from spinach.

The author is grateful to the American Academy of Arts and Sciences for a grant from the Warren Fund, which has greatly facilitated this study.

EXPERIMENTAL

Protoporphyrin Dimethyl Ester—Protoporphyrin was prepared according to Fischer and Pützer (10) by the action of iron on hemin in formic acid solution. Methylation with 1 per cent methyl alcoholic HCl gave the ester which after repeated recrystallizations from chloroform and methyl alcohol was in the form of microcrystalline prisms. Spectrum: I, 636–628; II, 589–585–571; III, 548–532; IV, 517–493 $m\mu$; IV, III, I, II.

Phyllin of Protoporphyrin Dimethyl Ester—Alkoxymagnesium bromide was used rather than the Grignard reagent because of the porphyrin carboxyl groups (11). Solid ethylmagnesium bromide from 1 mg. of magnesium was decomposed with 25 cc. of dry propyl alcohol. To the hot propoxymagnesium bromide was added 1 gm. of protoporphyrin dimethyl ester suspended in 200 cc. of hot propyl alcohol. After 15 to 30 minutes of refluxing, the porphyrin had dissolved to give a bright red phyllin solution with its characteristic two-banded spectrum. After 2 hours heating the solution was concentrated under reduced pressure, and the resulting oil taken up with ether, washed with aqueous ammonium chloride, water, and dried over sodium sulfate. Crystallization occurred from the concentrated solution on the addition of petroleum ether. The yield was about 1 gm. of phyllin. No solvent of crystallization was observed.

Protophyllin dimethyl ester dissolves readily in alcohol and acetone, less so in ether, to give bright red solutions with red fluorescence. It was recrystallized from ether-petroleum ether, and melted at 223–225° with sintering.

Analysis— $C_{38}H_{38}N_4O_4Mg$. Calculated, N 9.14; found, N 9.13, 8.90

Spectrum (in Ether)—I, 598–588–578; II, 562–549–535; III, 513–502 $m\mu$; I and II strong, III faint.

Pyridine Phyllochromogen of Protoporphyrin Dimethyl Ester—A solution of 200 mg. of phyllin in 10 cc. of ether containing 0.5 cc. of pyridine was slowly concentrated on the steam bath. Fine crystals with a dull gray sheen were deposited. These were filtered off after further concentration and addition of petroleum ether, washed with the latter, and dried by exposure to the air

at room temperature. The yield was 110 mg. In a melting point tube, sintering with unsharp melting occurred at about 134°.

Analysis—Calculated for a bipyridine complex; $C_{30}H_{38}N_4O_4Mg \cdot 2(C_5H_5N)$; C, 58.52, N 10.9. Found, 107.4 mg. substance lost after 12 hours in a vacuum at 100° 22.4 mg. or 20.85 per cent; 92.4 mg. substance lost after 2 hours 19.3 mg. or 20.89 per cent. Dumas nitrogen determinations gave 10.33 and 10.23 per cent.

Of the same sample, 48.4 mg. lost after 45 minutes at 100° in a vacuum 9.9 mg. or 20.4 per cent. The resulting 38.5 mg. of phyllin were left overnight in a vessel containing an open tube of pyridine; the sample gained 10.5 mg. or 21.4 per cent of the final weight.

Spectrum in Pyridine—I, 607–595–586; II, 573–560–543 $m\mu$. The faint band, III, of the phyllin has disappeared.

Protophyllchromogen is completely dissociated in ether without additional pyridine: I, 598–588–579; II, 564–550–534; III, 516–502 $m\mu$.

In a 1:1 mixture of pyridine and ether there is partial association: I, 599–593–587; II, 572–555–542 $m\mu$.

SUMMARY

1. There is described a new type of compound, phyllochromogen, formed by the addition of pyridine to porphyrin phyllin derivatives.

2. Points of similarity between such phyllochromogens and the state of chlorophyll in nature are discussed.

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THE HEME-GLOBIN LINKAGE OF HEMOGLOBIN

I. THE COURSE OF THE PANCREATIC DIGESTION OF OXYHEMOGLOBIN AND OF CARBOXYHEMOGLOBIN

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At four positions within the native globin molecule heme may combine to give a protein, capable of reversible combination with oxygen (1, 2) and identical with hemoglobin (3, 4). As many as thirty hemes may react with denatured globin, but the product cannot be reversibly oxygenated. Many other nitrogen-containing compounds combine with reduced hematin to form hemochromogens, but none of these combines with oxygen in this way. Particular interest is therefore attached to the nature of the union of heme to globin in native hemoglobin.

Analogies between hemoglobin and simpler derivatives have given some information about this point. Among the methods used have been electrometric titration, and the study of the magnetic properties, and oxidation-reduction potentials of the iron atom. More direct have been efforts to keep intact the original heme-protein linkage during degradation of the protein, chemical and enzymic hydrolytic agents having been employed. Waelsch (5) treated hemoglobin with 2 per cent sodium hydroxide at 85°, and isolated from the reaction a product containing hematin, proline, and alanine. By alcoholysis of hemoglobin at 190°, Küster and Koppenhöfer (6), also, obtained a proline-hematin fraction. No further support for the association of heme with proline has been presented. Haurowitz degraded hemoglobin with the tryptic (7) and papain (8) enzymes, both giving a product, "hemin proteose," rich in iron, with a molecular weight of "at

* Contribution No. 177.

least 23,000." On account of the large size of the molecule and the fact that the nitrogen distribution of the protein fragment was like that of hemoglobin, Haurowitz suggested that the prosthetic groups had split off and recombined with a large fraction of undigested globin. This proteose would have no relation to the original problem if this were true.

The present report concerns a study of the action of the pancreatic enzymes on oxyhemoglobin and on carboxyhemoglobin to determine the influence of carbon monoxide on the stability of the heme-protein linkage. The possibility of isolating the amino acid residues to which the heme was originally attached is presented if the linkage is so strengthened by carbon monoxide as to persist throughout the digestion.

Earlier reports indicated that CO hemoglobin was completely resistant to digestion by the pancreatic enzymes. Abderhalden and Damodaran (9) in 1930 found that both oxy- and CO hemoglobin were digested to nearly the same extent and at slightly different rates by pepsin-HCl, trypsin-kinase or erepsin, the differences being inconsistent, however. Haurowitz (7) found little difference between the splitting of the two derivatives even when CO hemoglobin was kept oxygen-free.

The present investigation has shown that oxy- and CO hemoglobin differ in the rate at which they are hydrolyzed by the pancreatic enzymes and also in the nature of the product formed from the prosthetic group. The data from such experiments are presented in Figs. 1 to 6. For reproducible results with CO hemoglobin it was necessary to remove all oxygen from the enzyme and substrate solutions before mixing and to keep the digest always free of oxygen.

At a hydrogen ion concentration of 8.0 (Fig. 1) the hydrolysis of CO hemoglobin differs from that of oxyhemoglobin by being slower in the earlier stages; after 5 days the degree of degradation is approximately the same for each. When hydrolysis with trypsin is complete, the ratio of amino to total nitrogen normally lies between 0.55 and 0.60. After hydrolysis with 20 per cent HCl, this ratio is 0.20 higher. At a lower pH, 6.2, (Fig. 2) the digestion of CO hemoglobin is greatly inhibited, whereas that of oxyhemoglobin proceeds almost unchanged. In these experi-

ments the ratio of protein to enzyme is the same (*cf.* also Fig. 3). In Fig. 4, at pH 7.8, the protein has been doubled in concentration and the enzyme decreased to one-thirteenth its former value. The difference in rates is still pronounced during the initial stages of the digestion, but the final hydrolysis reached is not so great

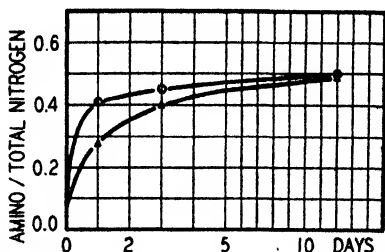


FIG. 1

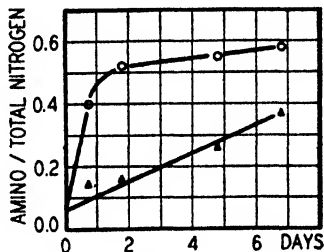


FIG. 2

FIG. 1. The pancreatic digestion of oxyhemoglobin (O) and of CO hemoglobin (Δ) at pH 8.0. The nitrogen concentration was 5.35 mg. per cc.; the nitrogen to enzyme ratio, 21.5.

FIG. 2. The pancreatic digestion of oxyhemoglobin (O) and of CO hemoglobin (Δ) at pH 6.2. The nitrogen concentration was 4.85 mg. per cc.; the nitrogen to enzyme ratio, 18.5.

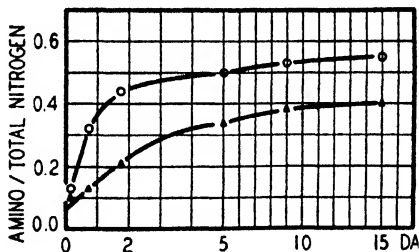


FIG. 3. The pancreatic digestion of oxyhemoglobin (O) and of CO hemoglobin (Δ) at pH 6; the nitrogen concentration was 7.35 mg. per cc.

as that with more enzyme. Thus, even at pH values near 8, the optimal point for pancreatic activity, the retarded digestion of CO hemoglobin is real, but with high concentrations of enzymes the difference might be overlooked.

The condition of hemoglobin during the digestions of oxyhemoglobin with trypsin has not yet been determined. Compara-

tive rate studies with reduced hemoglobin, methemoglobin, and CO hemoglobin are being made.

Although no instance was found of the inhibition of proteolytic enzymes by carbon monoxide, the possibility was considered. A gelatin solution was treated with carbon monoxide as in the hemo-

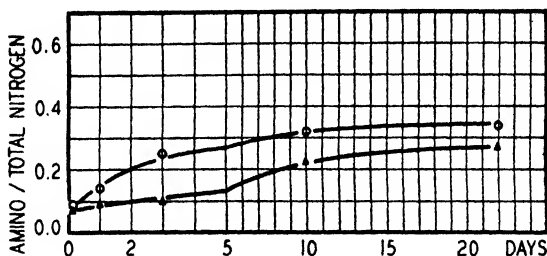


FIG. 4. The pancreatic digestion of oxyhemoglobin (O) and of CO hemoglobin (Δ) at pH 7.8. The nitrogen concentration was 11.0 mg. per cc.; the nitrogen to enzyme ratio, 450.

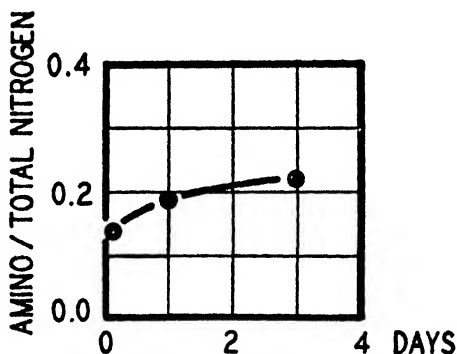


FIG. 5. The pancreatic digestion of gelatin in air (O) and of gelatin in CO (Δ) at pH 7.5. The nitrogen concentration was 7.25 mg. per cc.; the nitrogen to enzyme ratio, 48.

globin experiments; its hydrolysis with oxygen-free enzyme differed in no way from that without carbon monoxide (Fig. 5). More significant were similar digestions with globin, in which the relation of protein to enzyme corresponds to that with hemoglobin except for the fragments near the prosthetic group. Here, also, the hydrolyses of globin and of globin with carbon monoxide

were identical (Fig. 6). The effect of carbon monoxide in the hemoglobin experiments is therefore the result of its action on the substrate, not on the enzyme systems involved. Significant is the digestion of oxyhemoglobin run simultaneously with globin,—after 4 hours the liberated amino nitrogen was 8 per cent of the total nitrogen, whereas that from globin was 30 per cent of the total.

It is useful to consider the enzymic degradation of a native protein, such as hemoglobin, to consist of two related steps. In the first, the initiation of the reaction, the macrostructure of the protein is destroyed, giving rise to smaller fragments, the hydrolysis of which constitutes the final stages of the digestion. Peptide linkages may be split in both steps. Their location in

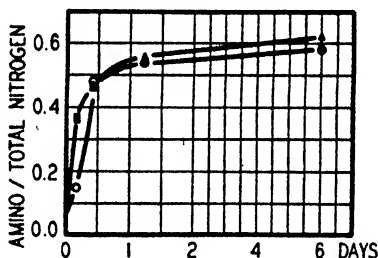


FIG. 6. The pancreatic digestion of oxyhemoglobin (O), of globin in air (□), and of globin in CO (Δ) at pH 6.3. The nitrogen concentration was 5.5 mg. per cc.; the nitrogen to enzyme ratio, 22.

a tightly knit and condense system of protein chains in the first stage, and, in simple polypeptide fragments in the latter, is the basis of differentiation. With conjugated proteins such as hemoglobin the prosthetic group may act as a binder, holding together extended protein chains through its association with specific amino acid residues within them.

In the present instance, it is clear that the hydrolysis of the smaller fragments is independent of the presence of carbon monoxide. The same peptides are formed from both hemoglobin and globin, and carbon monoxide does not retard their splitting with globin. The effect of carbon monoxide is expressed, however, through its retarding the rate at which these fragments are supplied.

The rôle of the four hemes in holding the molecule together is illustrated by the greatly retarded initial attack on the hemoglobin molecule, compared to that on the globin.¹ It is further developed by carbon monoxide, which, in strengthening the union of heme and globin, fortifies the interassociation of the protein chains and reduces the availability of their linkages to the enzymes.

The influence of carbon monoxide on the course of the reaction is further seen in the behavior of the prosthetic grouping during digestion. The term, prosthetic grouping, is used here to include the heme (ferrous derivative of protoporphyrin (10)), the amino acid residues to which it is attached in hemoglobin, and the associated gas, in this case carbon monoxide. Several hours after the addition of enzyme to oxyhemoglobin the solution takes a brown tone; within 24 hours it is an opaque brown liquid, having lost its former red color. At the completion of digestion a residue has formed on the bottom of the flask consisting of a brown flocculent precipitate and a bluish black microcrystalline powder. The former is similar to the "proteose" of Haurowitz (7), and is obtained in greater quantity by acidification of the hydrolysate solution. The crystalline fraction can be purified by washing with aqueous KHCO_3 , water, and alcohol and drying. It appears to be hematin; on reduction with hydrosulfite in the presence of pyridine, the pyridine hemochromogen spectrum is obtained. Soon after destruction of the oxyhemoglobin molecule has begun, the heme-protein linkage is weakened, and oxidation of the iron to the ferric condition occurs. There results separation of the prosthetic group as hematin or its dissociation and recombination with undigested protein. This would give a compound in its general nature like the cathemoglobin from the denaturation of methemoglobin.

The bright red color of CO hemoglobin remains unchanged during digestion if the solution is maintained free of oxygen. Little residue forms, except occasional deposits of tyrosine; no hematin or other crystalline porphyrin derivative is observed. Although the solution does not change its color on hydrolysis, a real shift in the position of the absorption bands occurs. Fig. 7 shows the absorption spectra of CO hemoglobin and of the carbon

¹ Configurational differences between native and denatured globin may also affect this point.

monoxide digestion product, obtained in the Hardy color analyzer.² The maxima for CO hemoglobin occur at 569 and 539 $m\mu$; those for the CO product at 564 and 535 $m\mu$. That both bands shift simultaneously 5 $m\mu$ to the blue and are not changed in relative intensity to one another would indicate that the chromophore group itself has not been altered; *i.e.*, that the prosthetic grouping has persisted from the native protein. Such a shift of the absorption region to shorter wave-lengths occurs commonly when the

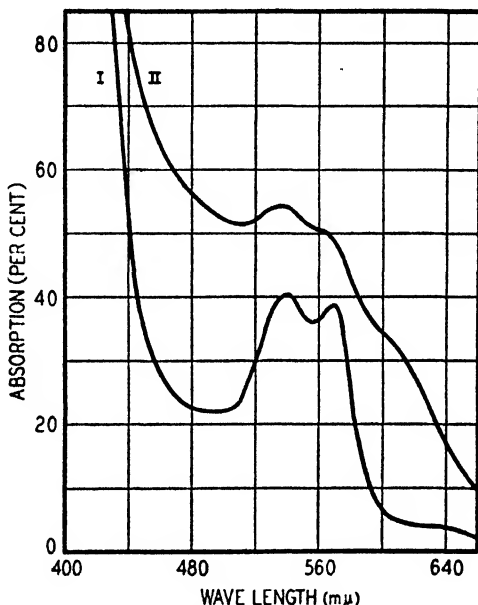


FIG. 7. Absorption spectra of CO hemoglobin (Curve I) and of the CO digestion product of hemoglobin (Curve II).

mass of the non-absorbing portion of a molecule containing a chromophoric group is decreased. It would therefore appear that most of the protein has been digested away, leaving the heme still associated with the amino acid residues to which it is combined in the original hemoglobin. This fragment is able to resist the

² These charts were made possible through the kindness of Mr. S. Q. Duntley and Professor A. C. Hardy of the Massachusetts Institute of Technology.

enzyme action through the protection, possibly steric, of the porphyrin group. The key to this is the carbon monoxide, which strengthens the bond of the heme, through its iron, to the protein.

It is a pleasure to acknowledge a grant from the Warren Fund of the American Academy of Arts and Sciences.

EXPERIMENTAL

Oxyhemoglobin—Horse plasma residues³ were diluted with 2 volumes of isotonic salt solution containing sodium citrate and passed through a Sharples centrifuge, kindly put at our disposal by Professor E. A. Hauser (11). From 8 liters of residues were obtained 6 liters of semicrystalline paste. Crystallization was furthered by adjusting the pH to 6.6 with 0.1 N HCl and stirring in an ice-salt mixture. The crystalline paste thus formed was washed with distilled water and stored under toluene at 0°. Residual stromata were removed by centrifugation of hemoglobin solutions before use.

Pancreatic Enzymes—100 gm. of pancreatin (Merck) were ground with 1 liter of 90 per cent glycerol and allowed to stand with frequent stirring for 2 days at room temperature. After the addition of 1 liter of distilled water, the solution was at once centrifuged free of residue and stored under toluene at 0°.

Digestion—The following typical procedure was that used in the experiments of Fig. 6. A concentrated solution of crystalline oxyhemoglobin was prepared with the aid of borate-HCl buffer (12), and centrifuged free of residue. A portion was transferred to a round bottom flask equipped with stop-cocks; the flask was evacuated and flushed with oxygen-free carbon monoxide. This procedure was repeated several times. Then there was drawn into the flask without the introduction of air the required enzyme solution, already flushed with nitrogen, and sufficient oxygen-free water to bring the nitrogen concentration to 5 mg. per cc. The flushing procedure was then repeated to insure removal of all oxygen.

Simultaneously another portion of the same hemoglobin solution was mixed with enzyme and water sufficient for the same nitrogen concentration.

³ Made available through the cooperation of the Massachusetts Anti-toxin Laboratory.

Amino nitrogen was determined by the Van Slyke volumetric method; total nitrogen by a semimicro-Kjeldahl procedure. A glass electrode was employed for pH determinations.

Globin—Globin was prepared by the acetone-HCl method of Anson and Mirsky (13). On account of its insolubility at pH 8 more acid solutions had to be employed. The solid product was powdered and ground with distilled water to give a solution of pH 2. This was carefully neutralized with dilute KOH to pH 5.5, when further addition of base caused a heavy precipitation. Treatment with carbon monoxide followed that used with hemoglobin. After mixing with enzyme, the pH was at 6.0, where it remained during the experiment.

SUMMARY

It is shown that the course of digestion of carboxyhemoglobin by the pancreatic enzymes is different from that of oxyhemoglobin.

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THE HEME-GLOBIN LINKAGE OF HEMOGLOBIN

II. THE MOLECULAR WEIGHT OF THE PRODUCT FROM CARBOXYHEMOGLOBIN

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Experiments reported in Paper I (1) led to the hypothesis that carboxyhemoglobin was digested by pancreatin without solution of the original heme-protein linkage of hemoglobin. Knowledge of the molecular size of the remaining fragment should either support or destroy this conclusion. Adair (2) by osmotic pressure experiments and Svedberg and Fåhræus (3) with the ultracentrifuge have shown the molecular weight of hemoglobin to be 67,000, four times the minimum molecular weight of 16,700 based on 1 iron atom per molecule. Our product from CO hemoglobin would appear to be the result of resynthesis, should it be sufficiently large to contain more than four hemes per molecule. In view of the dissociation of the hemoglobin molecule into half size fragments (Burk and Greenberg (4) and Steinhardt (5)) the more conservative figure of two hemes might be set, although it is not known that an even distribution of hemes occurs during dissociation. The molecular size, in addition to being helpful in the interpretation of the hydrolytic experiments, might also indicate the steric relation of the hemes to one another within the hemoglobin molecule.

Minimum Molecular Weight

The minimum molecular weight of the CO product was determined by its iron content. Upon the addition of acetic acid to the clear, red digest solution from CO hemoglobin, it becomes cloudy and a flocculent red precipitate separates. The latter

* Contribution No. 178.

may be collected by centrifugation, leaving a clear colorless mother liquor; it is washed with water, alcohol, and ether, and dried at 100° in a vacuum. Iron determinations, gravimetric and colorimetric (6), gave concordant results, the mean value being 5.24 per cent, which corresponds to a minimum molecular weight of 1060. Heme, 616, would constitute 58 per cent of the molecule, leaving 42 per cent for the protein fragment.

Example—72.5 mg. of dry CO product were ignited in a platinum crucible to give 5.6 mg. of ash. This was dissolved in HCl, filtered, precipitated with NH_4OH , collected on a filter paper, washed with 2 per cent NH_4NO_3 , and ignited, giving 5.4 mg. of ash, equivalent to 5.21 per cent iron. Again, 81.9 mg. gave 6.3 mg. of ash, equal to 5.38 per cent.

Product	Iron	Nitrogen
	<i>per cent</i>	<i>per cent</i>
159	5.36	12.23
168	5.39	12.64
179	5.28	12.24
183	5.17	
187	5.34	
222	5.28	12.50
352	5.07	

Purification by Dialysis

A pure solution of the unaltered product was desired for molecular weight determination. Although, under controlled conditions, the product may be flocculated with acetic acid, washed with oxygen-free water, and redispersed in dilute alkali without losing its color, the particle size appears to be increased. The red liquor so formed is cloudy, and under the microscope minute amorphous particles are visible.

Adequate purification was found in continuous dialysis (7) under an atmosphere of carbon monoxide with a cellophane membrane.¹ This permitted passage of the colorless degradation products but retained the heme-protein fragment complex. The course of dialysis may be seen from Table I. The fraction, retained during dialysis and precipitated by acetic acid, contains

¹ From the Central Scientific Company; wall thickness 0.00072 inch.

6 per cent of the total nitrogen and is equivalent to a single fragment of 1300 mass from 16,700 or 2600 from 33,400, etc., 12.5 per cent being used as the nitrogen content of the CO product.

The CO product purified by dialysis is unstable; addition of a trace of acetic acid results in complete flocculation from solution. Shaking for 30 seconds with air gives it a brown color, the red being completely lost within several minutes.

The absorption spectrum is unchanged after dialysis, the purified product having been used for the curve of Fig. 7 (*cf.* (1)). From the relation, $\beta = \alpha/cd$, where α is the fraction of light absorbed at the maxima of corresponding bands (λ 539 $m\mu$ for CO hemoglobin,

TABLE I
Dialysis of Pancreatic Digest of CO Hemoglobin

560 ml. of digest were taken. The rate of dialysis was 500 cc. per hour.

Fraction	Time	Nitrogen	Per cent of total	pH
	<i>days</i>	<i>mg.</i>		
Original.....		5065	100	5.8
Dialysate 1.....	5	4660	92.0	6.3
" 2.....	5	46	0.9	7.7
" 3.....	7	27	0.5	8.3
Residue.....	17	332	6.5	8.9
Pptd. by acetic acid from above residue.....		298	5.9	5.3

λ 535 for the CO product), c is the concentration of nitrogen in mg. per cc., and d the thickness of cell, there is found for the coefficient of absorption, β , 3.3 for CO hemoglobin and 54.5 for the CO product. These agree with the interpretation that much non-absorbing protein has been removed from the chromophoric group.

Osmotic Pressure Experiments

The osmotic pressure of this relatively pure solution of the CO digestion product was determined. A cell like that of Adair (2), modified to permit operation under carbon monoxide, was used. Table II contains the data from these experiments and

the molecular weights 2100, 2020, and 1240 calculated from the formula, $M = C/P \times RT$.

$$\text{At } 20^\circ, RT = \frac{0.08207 \times 76 \times 13.596 \times 293.1 \times 10}{100} = 2.486 \times 10^4$$

$$M = 2.486 \times 10^4 \times C$$

where C is the concentration per 100 cc. of solution, and P the pressure in cm. of water. A negative correction of 1.5 cm. has been applied to the observed value. This is the height of the meniscus when the same cell is filled with mother liquor from the acetic acid precipitation of the product. It is, therefore, a correction for capillarity and any high molecular material present.

TABLE II
Osmotic Pressure Experiments

Experi- ment No.	Solution	Time	Tempera- ture	Inner volume	Outer volume	P	Concen- tration of CO product	Mol. wt.
		<i>days</i>	<i>°C.</i>	<i>cc.</i>	<i>cc.</i>	<i>cm.</i>	<i>mg. per cc.</i>	
1	183	2	22.5	59	675	35.8	2.89	2180
2	188	5	20.0	59	683	37.8	2.95	2020
3	332	37*	20.0	60	55	46.5	2.25	1240

* With such a small outer volume, sufficient diffusion occurs for the concentration of the product outside to increase too rapidly for real maxima to be reached. This effect was overcome by changing the water outside until a true end-point was found. Six changes were required.

The behavior in Experiment 2, Table II, after the maximum had been reached is shown in Fig. 1. A final constant pressure was not obtained, but after 133 days the curve was nearly horizontal, at a pressure equivalent to a molecular weight of 16,300. The color of the solution within the membrane was unchanged after this time, and the outer liquid had just a faint pink tinge. This behavior was not explained until the CO product had been examined in diffusion experiments.

Diffusion

The method of Northrop and Anson (8, 9), in which a sintered glass membrane is employed, offered a simple means of studying

the properties and diffusion rate of the CO product. The standard apparatus was modified so that the working atmosphere could

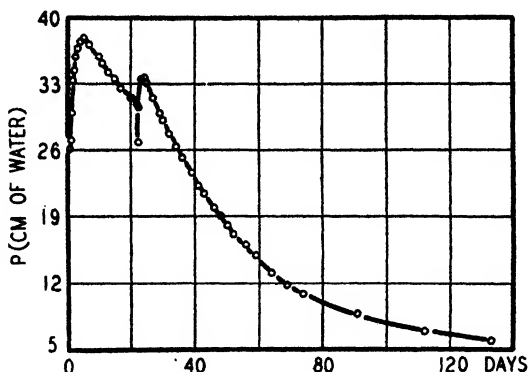


FIG. 1. The osmotic pressure of Experiment 2, Table II, over a period of 133 days. (The break after 24 days was caused by replacing the almost colorless outer liquid with distilled water.)

TABLE III

*Calibration of Jena Cell with 2 N NaCl at 25°; * Titration with 0.1 N AgNO₃; K₂CrO₄ As Indicator*

	Q	K cell	D
<i>days</i>			<i>sq. cm. per day</i>
0.0577	0.1496	0.486	1.26*
0.0417	0.1175	0.447	
0.0417	0.1205	0.436	
0.0417	0.1214	0.432	
0.489	1.350	0.455	
0.0625	0.1790	0.440	1.26
0.0625	0.1805	0.436	
0.0625	0.1837	0.429	
0.0554	0.1680	0.415	
Mean.....		0.442	

* Reference (9).

be controlled. Calibration of the Jena cell with 2 N NaCl at 25° is shown in Table III. The cell constant, K , was found by the use of the relation $K = Dt/Q$, where D is the diffusion co-

efficient of the substance in solution, and Q the cc. of inner solution that contain the amount diffused in the time t , expressed in days.

With the cell constant so obtained, the diffusion constants for sucrose, raffinose, and CO hemoglobin were determined. These values are shown in Table IV with corresponding figures from other sources.

In Table V are the diffusion coefficients, D , of the CO product under various conditions,—also the corresponding molecular weights. Undiluted CO product solution was used in the diffu-

TABLE IV
Diffusion Constants for Sucrose, Raffinose, and CO Hemoglobin
 K cell = 0.442.

	t	Q	D found	D known
	<i>days</i>		<i>sq. cm. per day</i>	<i>sq. cm. per day</i>
Sucrose, 1 N aqueous solution, 25°	0.108	0.0878	0.361	0.380*
	0.166	0.1393	0.371	
	0.125	0.1007	0.356	
	0.146	0.1190	0.362	
Raffinose, 0.125 gm. per cc., 24.5°	0.236	0.1820	0.341	0.344*
	0.156	0.1211	0.342	
	0.464	0.3524	0.336	
	0.281	0.2152	0.339	
COHb in borate-HCl, pH 8.3, 25.5°	0.625	0.0765	0.054	0.068†
	1.07	0.153	0.063	0.077‡
	0.930	0.162	0.077	

* Reference (10).

† Calculated from $D = 6.9 \times 10^{-7}$ sq.cm. per second at 20° (11).

‡ Calculated from $D = 0.042$ sq.cm. per day at 5° (8).

sions against water, with the single exception of Experiment 6 in which the solution was diluted with an equal part of water. The borate-HCl and glycine-NaOH buffers were prepared in twice the strength of Bertho and Grassmann (12), being diluted with an equal volume of CO product for the inner and of water for the outer solution. The procedure of Northrop and Anson was followed, a 2 hour period being allowed for the system to reach equilibrium. Diffusion was followed colorimetrically by comparing the diffusate with a diluted solution from within the cell after the CO product had become oxidized

brown. The properties and previous history of each solution are summarized in Table VI.

The molecular weights of Table V were calculated by the equations, $D = RT/N6\pi\tau\eta$, and $M = \frac{4}{3}\pi r^3 g N$, which combined and evaluated give for the molecular weight at 25, $M = 30.84/D^3$.*

TABLE V
Diffusion of CO Product

Water thermostat at 25.0°.

Ex- peri- ment No.	Pro- duct	Solvent	K cell	D	Mol. wt.
				<i>sq. cm. per day</i>	
1	230	Water	0.110*	0.42, 0.43, 0.44, 0.36, 0.46	420
2	184	"	0.685†	0.39, 0.47, 0.43	390
3	184	"	0.442‡	0.444	365
4	183	"	0.442‡	0.389, 0.400	500
5	332	"	0.442‡	0.365	635
6	332	1:1 water	0.442‡	0.405, 0.464, 0.541	300
7	230	Borate-HCl, pH 8.3§	0.442‡	0.072, 0.092	55,900
8	184	Glycine-NaOH, pH 11.6§	0.442‡	0.075, 0.092	55,900
9	183	Same	0.442‡	0.099	31,000
10	183	0.01 N KOH, pH 11.5	0.442‡	0.199, 0.205, 0.199	3,860
11	197	Same	0.442‡	0.091, 0.112	31,000
12	184	0.015 N KOH	0.442‡	0.165, 0.167	6,740
13	332	Same	0.442‡	0.133, 0.135	12,800
14	332	1.25 N KOH	0.442‡	0.040, 0.047	388,000
15	188	¶	0.442‡	0.127, 0.143	13,300

* Alundum disk.

† Jena cell before grinding.

‡ Final Jena cell (Table III).

§ Reference (12).

¶ Diffusion of 188 after 133 days in osmotic pressure experiment of Fig. 1; against outer liquor from the same experiment.

The equation of Einstein and of Sutherland, $D = RT/Nf$, is restricted to uncharged molecules much larger in size than the

* $R = 8.3 \times 10^7$ erg, degree⁻¹, molecule⁻¹; $T = 298^\circ$; $N = 6.06 \times 10^{23}$ molecule⁻¹; $\eta = 0.00895$ erg, second, cm.⁻²; $g = 1.3$; f , the friction coefficient, $= 6\pi\tau\eta$ for a sphere.

molecule of solvent. Its applicability is therefore restricted here; the CO product is undoubtedly charged, and its molecular size is relatively small, especially in those experiments in which salt-free water was used as solvent.

Dilution of the CO product with buffer or basic solutions was in every case accompanied by a slight clouding up of the solution, indicative of increased Tyndall intensity. This phenomenon did not accompany dilution with water (Experiment 6). Freshly dialyzed CO product was also unstable in this way; in Experiment 13 a solution was taken directly from dialysis for diffusion in 0.015 N KOH. It is believed that the aggregation of the meta-stable CO product into larger colloidal particles causes this, a

TABLE VI
CO Product Solutions Studied

Product	Nitrogen	pH	Period dialysed
	<i>mg. per cc.</i>		<i>days</i>
183	0.55	8.8	5.5
184	0.45†	9.0	5
188*	0.36	9.0	10
197	0.43	8.9	17
230	0.67	9.0	7
332	0.89	9.4	7

* Solution 184 after 5 days additional dialysis.

† Calculated from values for 188 and the dialysate.

belief which is supported by the low diffusion constants, paralleling the observed increase in Tyndall effect. The instability of the dialyzed solution is doubtless due to the absence of the stabilizing influence of protein hydrolytic products. Only those diffusion constants in salt-free water are accepted as relating to the original unaggregated form of the CO product. That the CO product also slowly aggregates in salt-free solutions is shown by the continual fall in osmotic pressure after a maximum has been reached (Fig. 1, and Table V, Experiment 15). The CO product after 133 days osmosis was transferred directly to the diffusion cell and its rate of diffusion against the outside osmotic liquor determined. The red inner solution was very cloudy, and some residue was present on the bottom of the cell. The maxi-

mum osmotic pressure of this solution had been reached after 5 days and corresponded to a molecular weight of 2020; at this time the molecular weight calculated from diffusion against water would lie near 450 (Table V). After 133 days the osmotic pressure was equivalent to a molecular weight of 16,300, and now its diffusion constant equaled 13,000.

DISCUSSION

The methods of diffusion and osmotic pressure do not give wholly satisfactory results under the best conditions. With such an unstable material as CO product the results may be subject to question, but, in the absence of better information, must be considered.

In the classical osmotic studies of Adair on hemoglobin it was found that reproducible values were obtained with buffered solutions at the isoelectric point (13) or in salt-free solutions (14). The tendency of the CO product to aggregate on dilution with electrolyte solutions excludes experiments at the buffered isoelectric point; simultaneously are eliminated complications from the Donnan effect. The salt-free solution from dialysis should give acceptable values in the absence of high molecular fragments and of ionic forces. The first factor may be excluded by the low pressures of the mother liquor after acetic acid precipitation of the product. Adair's acceptable values with salt-free hemoglobin indicate that ionic forces are negligible with the almost neutral protein. One hesitates to extend this, however, to the CO product, whose purified solution has a pH of 9, although the alkalinity may not be contributed by the product itself.

The diffusion constant of the CO product against electrolyte-free water is high, the calculated molecular weights being correspondingly low. The average value found was 0.42 sq. cm. per day, comparable with 0.38 sq. cm. per day for sucrose.

The effect of coulomb or ionic forces on diffusion rates has been treated by Bruins (15), Hartley and Robinson (16), and Heidelberg and Kendall (17). Hartley and Robinson were concerned with colloidal electrolytes, in particular dyes. The increased charge resulting from aggregation of such particles effects an increase in the diffusion rate contrary to that to be expected from the Stokes-Einstein equation. Addition of readily diffusible

electrolytes to the solution reduces this potential gradient, giving a true value only when the conductance of solution is sufficiently high to destroy it completely. The original high diffusion rate of our CO product in electrolyte-free solution may be due to such a charge on the particle and the electrical gradient thus set up. Aggregation has, however, a decidedly different effect. When this occurs as a result of long standing (Experiment 15, Table V), without addition of electrolytes, the diffusion constant falls. The electrical gradient is not enhanced, but a neutralization of charges occurs. After this process has taken place, the molecular weights from osmosis and diffusion agree (16,000 and 13,000). The effect of added electrolytes is hardly analogous to that discussed by Hartley and Robinson. The solution shows a Tyndall increase as it does under salt-free aggregation, and the discordant values for the diffusion constants give molecular weights from 6000 to 400,000, with no limiting figure.

Ionic forces may therefore be considered a real factor here in determining the rate of diffusion. Diffusion across such a permeable membrane as that used is treated as if actuated by the gradient osmotic pressure within the cell. Accordingly the osmotic pressures observed with the CO product also must be viewed with the possibility of ionic interference.

In spite of these factors which have to be considered in interpreting the experimental data, there is general agreement in the figures from different sources. The minimum molecular weights from iron content, 1060, and from residual weight after dialysis, 1300, and the figures from osmosis, 2180, 2020, and 1240, indicate a value acceptable for a one or two heme fragment. The values from diffusion constants also are in accord with a relatively small weight. The agreement between osmotic and diffusion figures after aggregation (13,000 and 16,000) shows that the original product cannot be of this magnitude. The possibility that the CO product is a result of resynthesis formed from the recombination of many hemes with a large fragment of undigested protein is thus discredited. Were the mean value of 450 off by a factor of 10, the result would still permit a four heme fragment; even this factor would appear too great from the data at hand.

These studies have therefore not only failed to produce evidence against the theory that pancreatic CO hemoglobin digestion

proceeds without rupture of the original heme-globin linkage, but make it appear likely that the resulting fragment contains one or two heme groups associated with a polypeptide of 500 or 1000 size, respectively.

A true determination of the particle size of the CO product is a desirable objective as a means of determining the position of the four heme residues within the native hemoglobin molecule. Should this fragment contain two hemes, which is most probable from our data, strong evidence would be given that the hemes are placed in pairs along single protein chains with only 8 to 10 amino acid residues between them. Each of the half molecules of Burk and Steinhardt would thus contain a heme pair. On the other hand a figure of four minimal weights would support uneven distribution of the hemes between the half size molecules. It is hoped that the study of the amino acid residues present in the protein fragment, which is now in progress, will give more positive proof for or against the two heme figure.

SUMMARY

Minimum molecular weights for the product from pancreatic digestion of CO hemoglobin are 1060, based on the iron content, and 1300, on the non-dialyzable residue.

Data from osmotic pressure determinations and from diffusion experiments are in agreement with a molecular weight of once or twice this value.

The product appears to contain the original heme-protein linkage of hemoglobin.

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CAROTENE

XI. ISOLATION AND DETECTION OF α -CAROTENE, AND THE CAROTENES OF CARROT ROOTS AND OF BUTTER

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Contradictory reports concerning the α -carotene content of butter (1-3) have prompted a reexamination of the methods for estimation of carotenes. This has led to a redetermination of the carotenes of carrots, of leaves, and of butter obtained from cows fed various proportions of carotene isomers.

The most sensitive and specific method for the detection of α -carotene is chromatographic adsorption. With specially prepared magnesia as adsorbent (to avoid alteration of the carotenoids by adsorption and to eliminate losses by crystallization) (1, 4) it has been possible to demonstrate changes in the α -carotene content of butter not detectable by other methods.

The occurrence of α -carotene in butter is dependent upon the α -carotene content of the rations fed the cows. The presence in butter of other carotene isomers and of the colorless, weakly adsorbed, fluorescent substances found in leaves (5) likewise depends upon the quantities of these materials contained in the rations of the animals.

Convenient sources of carotene isomers and of the weakly adsorbed, fluorescent leaf constituents for feeding experiments are carrots and extracts of carrots. In addition to α -, β -, γ -, and δ -carotene, carrots contain relatively large amounts of a carotene with spectral absorption properties similar to those of the flavoxanthins (6-10). This flavoxanthin-like carotene, which does not occur in appreciable quantities in leaves, and which is isolated readily by adsorption of saponified carrot extracts, is easily detectable in butter by spectrophotometric methods.

Methods

Two independent methods have been utilized for determination of the carotenes of leaves, carrots, and butter; namely, chromatographic adsorption (magnesia) (1, 4) and spectral absorption (photoelectric) (11). Spectral absorption properties of pure chemical compounds are usually represented by plotting the logarithm of the molecular extinction coefficient $\log E = \log [(\log I_0 - \log I) \div Lc]$ against the wave-length. I_0 and I are, respectively, the transmission of light by L cm. of solvent and solution and c is the concentration of pigment in moles per liter of solution. In case the pigments cannot be isolated in a high state of purity, considerable information concerning the properties of the mixture may be obtained from the shape of the so called characteristic absorption curve, the plot of $\log (\log I_0 - \log I)$ against the wave-length (12). Alteration of the composition of the pigment mixture is accompanied by change in shape of the characteristic absorption curve.

In order to compare the absorption properties of pigment mixtures contained in natural products such as leaves and butter with those of pure pigments, it is convenient to express the absorption as $\log s = \log [(\log I_0 - \log I) \div Lc]$ where c is expressed in gm. of material (e.g., butter) per liter of solution ((8) p. 125). Such curves have the same shape as the characteristic absorption curves, and, when the extinction coefficients of the pure pigments are known, the concentration of each pigment in the mixture may be calculated. Comparison of the shapes of different absorption curves is facilitated by addition of a constant, k , to the values of $\log E$ or $\log s$.

Results

Separation of α - and β -Carotene by Adsorption—The minimum quantity of α -carotene that can be detected in the presence of β -carotene varies with the size of the special magnesia (4) adsorption columns and with the amount of the mixture adsorbed. With columns 0.7 cm. in diameter it is possible to isolate α -carotene from mixtures containing as little as 0.02 per cent of the α isomer provided 9 to 10 mg. of the mixture are adsorbed.

Carbon tetrachloride may be used instead of petroleum ether as a solvent for separation of carotenes by adsorption. The

adsorbed carotene is eluted completely with ethanol, and about 90 per cent of the separated carotenes is recoverable in crystalline form.

The reaction between magnesia and adsorbed β -carotene was followed spectroscopically. Equal quantities (5 mg.) of nearly pure β -carotene were adsorbed from various solvents upon columns 1.2 by 10 cm., composed of magnesia and siliceous earth (1:1). After some time, the pigments were eluted with 30 ml. of solvent containing a little ethanol. This solution was diluted with ethanol and the absorption of the pigments (based upon the

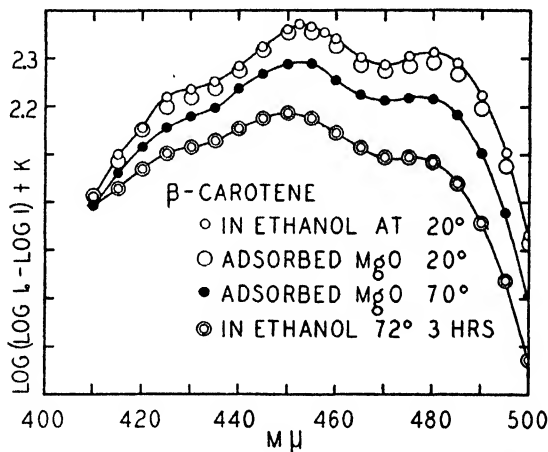


FIG. 1. Characteristic absorption curves of β -carotene adsorbed on magnesia from *n*-heptane and of β -carotene heated in ethanol. Solvent, ethanol.

quantity of carotene originally adsorbed) was compared with that of the unadsorbed carotene (Fig. 1). At 20° β -carotene is altered but slightly by adsorption on magnesia for 1.5 hours from *n*-heptane, and about 97 per cent of the pigment is eluted with ethanol. At 70° adsorption on magnesia for 1 hour from *n*-heptane causes decomposition of the carotene similar to that which occurs when solutions of carotene are heated (Fig. 1). Similar results were obtained when carbon tetrachloride was used as solvent.

Decomposition of β -carotene in ethanol by the action of heat

is illustrated by Fig. 1. α -Carotene is decomposed in a similar way when heated in sealed tubes with pyridine, but it is not converted into β -carotene, as shown by chromatographic adsorption of the products.

α -Carotene is also decomposed by very strong alkalies such as a solution of potassium amide in liquid ammonia but it is not converted into β -carotene.

Carotenes of Carrots and of Leaves—Carrot roots that had been in storage for some time (27.3 kilos) were sliced, extracted with water (13), dried in air, reduced to a fine powder in the ball mill, and extracted with petroleum ether (5 liters) which was subsequently concentrated to about 750 ml. This concentrate was treated at 20° with potassium hydroxide (50 gm.) dissolved in methanol (250 ml.). Petroleum ether (1.5 liters) and water (100 ml.) were added to the carotene solution and the aqueous layer which separated was removed. The petroleum ether layer was extracted repeatedly with methanol (90 per cent), then with water, and concentrated to about 250 ml. This solution was passed through a magnesia adsorption column (Column I) (6.7 by 37 cm.) (4) which was then washed with petroleum ether until the α - and β -carotene were carried through. The percolate containing the α - and β -carotene was collected in portions of about 150 ml., and these were passed successively through another column 6.7 by 37 cm. In this way the α - and β -carotene were separated from each other. No indications of an isomerization of α -carotene were observed (3). Relatively very large quantities of colorless fluorescent substances similar to those found in leaves (5) were adsorbed below the α -carotene. These materials were isolated as oils which oxidized rapidly in air and did not crystallize from methanol. Below the fluorescent compounds on the adsorption column there appeared small quantities of a carotenoid pigment which resembled a pigment previously separated from palm oil (4) and from the tops of the white carrot (1).

Column I, after having been washed with petroleum ether as described already, contained four definite bands of adsorbed carotenes. Near the top of the column was a rather indefinite orange-red band about 3 cm. wide (Band I). Near the center of the column was a red-orange band about 3 cm. wide (Band II). Below this band was a white space about 1 cm. wide and

below this was a yellow band about 2 cm. wide (Band III). The lower 12 cm. of the column were lemon-yellow (Band IV). The pigment from Band I (eluted with ethanol) did not exhibit well defined absorption maxima and minima. The characteristic absorption curve of the pigment from Band II (λ maxima 435, 460, and 490 $m\mu$ in petroleum ether) indicated that this carotene was identical with γ -carotene (14). The pigment from Band III exhibited absorption maxima at 428, 454, and 484 $m\mu$ and absorption minima at 439 and 474 $m\mu$, indicating that it was δ -carotene (15). Since considerable quantities of the pigment from Band IV were present, the solution of the eluted pigment was concentrated, and the pigment itself was crystallized by the addition of methanol to the concentrated solution. These crystals, which were orange-colored, contained colorless impurities which did not dissolve in acetone. The characteristic absorption properties of a solution of the crystals in petroleum ether are shown in Fig. 2. This carotene exhibits many reactions characteristic of the carotenoids. It is oxidized very rapidly to colorless substances upon exposure to air. It is much more soluble in petroleum ether than in 90 per cent methanol. With sulfuric acid and with antimony trichloride in chloroform it forms blue solutions which do not exhibit definite absorption maxima. In ether solution, the carotene does not form colored products with concentrated hydrochloric acid. The wave-lengths of the absorption maxima of this pigment in solution in carbon disulfide are 423 and 454 $m\mu$. This is considerably different from the wave-lengths of the absorption maxima of a strongly adsorbed carotene separated from carrots by Karrer, Schöpp, and Morf (10). The pigment appears to be identical with a carotene first detected in carrots by the spectroscopic investigations of van Stolk, Guilbert, and Péneau (9).

All the substances described in the preceding paragraphs were also isolated from a technical preparation of carotene dissolved in cottonseed oil. This carotene, which was fed to cows as described in the following section, was prepared by concentration of an extract of carrots; hence loss of carotene isomers by crystallization was avoided.

Characteristic absorption properties of carotene mixtures obtained from various plant parts are reported in Table I. About

20 gm. of plant material were extracted with ethanol. Fats and chlorophylls in the extracts were saponified with potassium hydroxide and the carotenoids were transferred to petroleum ether, which was then extracted repeatedly with methanol (90 per cent) in order to remove the xanthophylls. Characteristic absorption properties of the petroleum ether solutions were then determined.

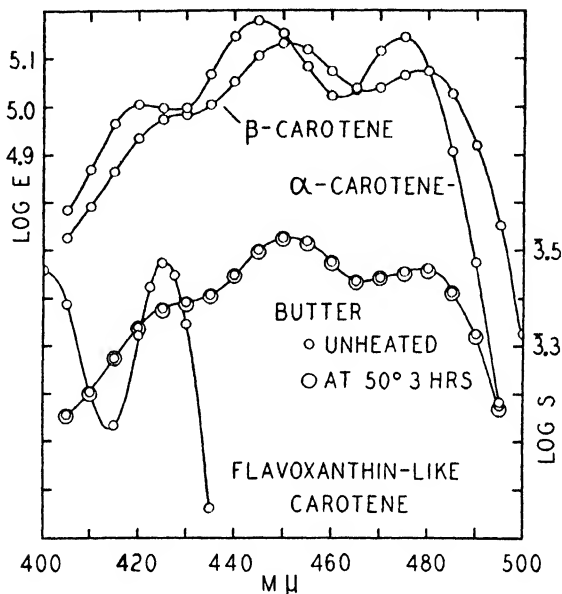


FIG. 2. Extinction coefficients of α - and β -carotene; values at left of figure. Characteristic absorption curve of the flavoxanthin-like carotene from carrots and specific absorption curve of butter (from Cows A and B) before and after heating; values at right of figure. Solvent, petroleum ether.

For comparison with β -carotene, the values of the characteristic absorption values have been made equal at 480 $m\mu$, the wavelength at which the absorption values of α - and β -carotene are equal (Fig. 2). It is only the carotenes from carrot roots that contain large quantities of the pigment which exhibits strong absorption at short wave-lengths.

Extinction Coefficients of α - and β -Carotene—The molecular extinction coefficients of α - and β -carotene, dissolved in petroleum

ether (b. p. 55-65°), are recorded in Fig. 2. These results are in moderately good agreement with absorption coefficients reported by Peterson, Hughes, and Freeman (16) and by Clark and Gring (17).

Carotenes of Butter—When Jersey cows were fed rations (beet pulp, alfalfa pasture, and alfalfa hay) containing β -carotene as the

TABLE I

Characteristic Absorption Properties of Carotene from Various Sources; Solvent, Petroleum Ether

The results are expressed in $\log s + k$ as defined under "Methods."

Wave-length $m\mu$	Carrot				White carrot leaves	Sun- flower leaves	Tobacco leaves	Incense cedar branch- lets
	Tip of roots	Centers of roots	Crowns of roots	Leaves				
405	4.874	4.842	4.919	4.755	4.737	4.740	4.750	4.767
480	5.073	5.073	5.073	5.073	5.073	5.073	5.073	5.073
490	4.838	4.839	4.825	4.865	4.892	4.902	4.891	4.823

TABLE II

Carotene (α Plus β) Content of Butter (Calculated from Absorption at 480 $M\mu$) and α - and β -Carotene Content of Butter (Determined by Absorption after Separation of the Pigments by Adsorption)

Per 100 gm. butter	Cows A and B	Cow A		Cow B	
	Alfalfa, beet pulp	Beet pulp, cottonseed	Carrots, beet pulp, cottonseed	Beet pulp, cottonseed	Carotene, beet pulp, cottonseed
	mg.	mg.	mg.	mg.	mg.
Carotene.....	1.32	0.59	0.85	0.41	0.51
α -Carotene.....	0.0043		0.069		0.043
β -Carotene.....			0.45		0.206

principal constituent of the carotene mixture for more than 30 days, the butter carotene was composed almost entirely of β -carotene (Fig. 2; Table II). Petroleum ether-soluble, fluorescent compounds which are adsorbed below α -carotene (6) were not detectable by adsorption. The same cows given rations containing reduced quantities of carotene (beet pulp and cottonseed meal) for the following 19 days produced butter containing small

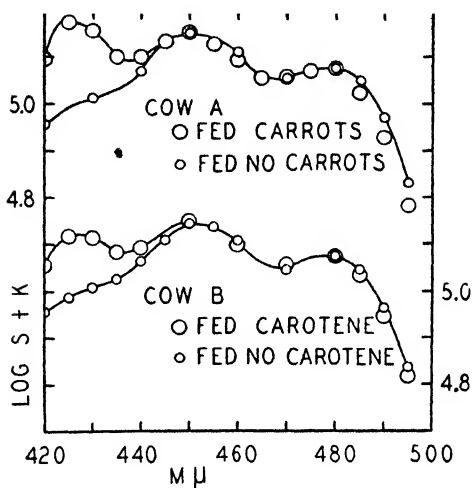


FIG. 3. Characteristic absorption curves of butter before and after feeding Cow A carrots and Cow B carrot root carotene. Solvent, petroleum ether.

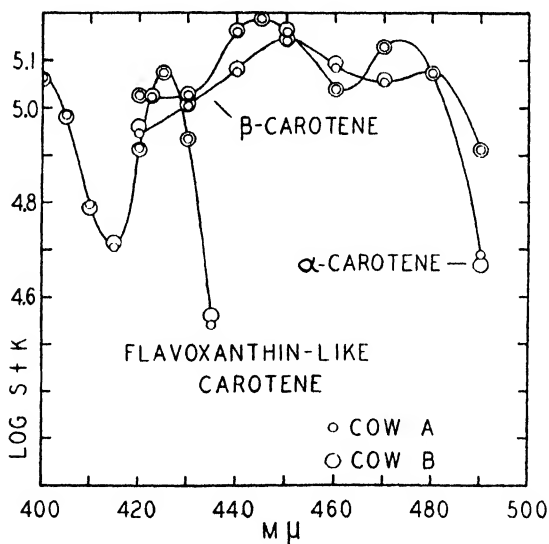


FIG. 4. Characteristic absorption curves of carotenes isolated from butter after feeding Cow A carrots and Cow B carrot root carotene. Solvent, petroleum ether.

quantities of carotene consisting principally of the β isomer (Fig. 3; Table II). Addition to the rations of substances rich in α -carotene, the flavoxanthin-like carotene, and the fluorescent substances referred to above resulted in the excretion of all these substances in the butter, as indicated by increased absorption of the butter at short wave-lengths (Fig. 3) and as proved by isolation of the pigments by adsorption followed by determination of their characteristic spectral absorption (Fig. 4; Table II). (Cow A received carrots with tops, 150 pounds for 3 days, and carrots, 370 pounds for 4 days. Cow B received beet pulp, cottonseed meal, and approximately 2 gm. of carotene in cottonseed oil daily for 9 days.¹)

For determination of the absorption properties of butter, the freshly churned fat was melted at 50° and permitted to stand 1.5 hours in order to remove the buttermilk. This rendering did not alter the butter carotene (Fig. 2). Samples of the rendered fat (6 to 12 gm. dissolved in petroleum ether, filtered, and diluted to 100 ml.) were used for the spectrophotometric measurements.

For separation of the butter carotenes by adsorption, rendered butter (about 500 gm.) was dissolved in twice its volume of absolute ethanol and allowed to stand at 2-5° overnight. Crystals which separated were removed, washed, and the filtrate treated with a large excess of potassium hydroxide dissolved in methanol. After several hours, the pigments were transferred to petroleum ether which was extracted with methanol (80 per cent) and with water, dried, concentrated to about 10 ml., and adsorbed on magnesia and siliceous earth columns 2 by 13 cm. The quantities of carotene isolated from the column (Table II) include the losses due to removal of the colorless materials, to partition, and to adsorption.

DISCUSSION

Estimation of the various carotenoids in natural products depends upon utilization of methods which do not alter the relative proportions of these pigments and which do not decompose the pigments themselves. When these precautions are taken, analy-

¹ These feeding experiments were carried out under the supervision of Dr. H. R. Guilbert, at the Department of Agriculture of the University of California at Davis.

sis of carotenes from carrots reveals the presence of a pigment which exhibits an absorption spectrum similar to that of the flavoxanthins. Since this substance has not yet been isolated in a high state of purity, the exact quantity present in carrots cannot be determined, but, if one assumes that the extinction coefficients of this pigment at maximum absorption are equal to those of α -carotene at maximum absorption, then the amounts of these two pigments must be approximately equal. The absence of this flavoxanthin-like pigment from carrot and sunflower leaves, which vary greatly in α -carotene content, indicates that its presence in natural products is not proportional to the α -carotene content.

Since the carotenes of butter vary with those ingested by the animals, investigations of the butter pigments must be supplemented by investigations of the carotenes of the rations. Occurrence of only β -carotene in butter after cows had received principally this pigment in the rations indicates that carotenes are not interconvertible in the cow. The proportions of the carotene isomers isolated from butter after cows had been fed carrots were similar to the proportions of the carotenes in the rations. The rapidity with which carotenes of carrot roots are transferred to butter suggests that carrots may be used as a source of vitamin A in milk and butter in northern and arid regions when green fodder is not available.

It is a pleasure to acknowledge many helpful suggestions made by Dr. H. A. Spoehr and Dr. J. H. C. Smith and the assistance rendered by Dr. H. R. Guilbert in supervising the feeding experiments and in collecting the butter.

SUMMARY

Carrots contain considerable quantities of a carotene pigment the spectral absorption properties of which are similar to those of the flavoxanthins. This pigment does not occur in appreciable amounts in leaves. Carrot roots are also an excellent source of the colorless, weakly adsorbed, strongly fluorescent substances first isolated from leaves.

The carotenes of butter are dependent upon those contained in the rations of the cows. The weakly adsorbed, fluorescent constituents of ingested carrots also appear in the butter fat.

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A NEW METHOD FOR THE MICRODETERMINATION OF MANGANESE IN BIOLOGICAL MATERIALS*

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In our studies on perosis in chicks, it was found desirable to be able to determine small amounts of manganese of the order of 0.1 to 10 micrograms. Such a method was necessary for the determination of manganese in bones, blood, and similar materials. The formaldoxime method of Sideris (1) was found unsuitable, as the determination is carried out in alkaline solution. Under these conditions phosphates precipitate, causing a masking and adsorption of the color. The standard method in which the manganese is oxidized to permanganate and the color thus developed compared with a standard was not sufficiently sensitive. The smallest amount detectable with the use of an Evelyn photoelectric colorimeter and a 5200 Å. filter was 25 micrograms in 25 cc.

In 1913 Dietz (2) and later Feigl (3) and Olszewski (4) described a very sensitive qualitative test for manganese. In alkaline solution manganese dioxide and the permanganate ion oxidized benzidine to give a blue color. With this test it was possible to detect 1 part of manganese in 250,000,000. Stratton and coworkers (5) in 1932 and Clark in 1933 (6) suggested the use of benzidine for the quantitative determination of permanganate. However, the blue color obtained was found to fade in 2 minutes, thus seriously limiting the method.

Fundamentally, the method depends on the oxidation of benzidine. In dilute solutions of oxidizing agents the merquinoidal

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type of compound (7) is formed, which is green in acid and blue in neutral solution (8). When a neutral solution of dilute permanganate was added to a solution of purified benzidine in 5 per cent acetic acid, a deep blue color developed. Attempts to evaluate the color by means of the Evelyn photoelectric colorimeter and a 6600 Å. filter met with failure, as it was found that the color developed started to fade immediately on formation. Thus accurate estimations of the small amounts of manganese present were impossible.

TABLE I
Stability of Color for Various Amounts of Manganese

Time min.	Galvanometer deflection		
	1 microgram	5 micrograms	10 micrograms
1	75 ¹	40 ²	16 ³
2	74 ³	35 ¹	14
3	74 ¹	33 ³	12 ³
4	74 ¹	33	12 ¹
5	74 ¹	32 ³	12
6	74 ¹	32 ³	12
7	74 ³	32 ³	12
8	74 ³	32 ³	12
9	74 ³	32 ³	12
10	75	32 ³	12
11	75	32 ³	12 ¹
12	76	33	12 ¹
13		33	12 ³
14		33 ¹	12 ³
15			12 ³

However, when solutions of benzidine were added to permanganate solutions made strongly acid with nitric acid, a yellow-green color instead of the blue was obtained. In tests of this color with the photoelectric colorimeter, with a 4200 Å. filter, it was found to be a great deal more stable. Table I shows the relative stability of the color developed with various amounts of manganese. From these data it is seen that the maximum color develops in from 3 to 5 minutes and that the color is stable for 5 minutes.

It was decided to carry out the determination in nitric acid solution. Sulfuric acid could not be used, as sulfate forms a precipitate with benzidine, and hydrochloric acid was also undesirable, as it reduces permanganate ion and yields free chlorine in the presence of oxidizing agents.

As other oxidizing agents give the same color with benzidine (2), this reaction is not specific for permanganate. In the determination manganous ion must be oxidized to permanganate ion, and the reagent chosen for this oxidation must be one which will not leave any excess oxidizing agent to come in contact with the benzidine. For this purpose sodium bismuthate was selected, as it readily decomposes in acid solution, leaving insoluble bismuth oxide, which can be easily filtered off.

The following metals, cerium, cobalt, nickel, thallium, and silver, as their hydrated oxides have been found by Feigl (3) to give the same blue color with benzidine. When salts of these metals were used in our regular procedure, no color was obtained. Ferric iron (3) and copper were also found not to interfere with the determination.

However, chlorides (and other halides) do interfere by the formation of free chlorine (5) on treatment with the oxidizing agent and hence it is necessary to remove them from the sample. Silver nitrate was used, but here again trouble was encountered. The excess silver ion reacts with sodium bismuthate to form an unstable complex of silver bismuthate which is soluble in dilute nitric acid solution (9). This allowed some oxidizing agent to pass through the filter and react with the benzidine, thus yielding high results. We have also observed a similar effect with cobalt. However, with concentrations of cobalt up to the order of 20 times that which would ordinarily be found in biological material no interference was encountered. It is necessary, therefore, to remove the chlorides by adding an excess of nitric acid and evaporating to dryness on the hot-plate, care being used to prevent spattering. Two to three evaporations are sufficient to free the sample from chlorides.

The nitric acid used must be free of oxides of nitrogen, as these react with benzidine to form interfering colors. These oxides are easily eliminated when the nitric acid is boiled with sodium bismuthate.

Another difficulty encountered was the fading of the permanganate after filtering off the sodium bismuthate and before adding the benzidine. It was found simpler to filter directly into the colorimeter tubes containing the benzidine solution, and thus no loss occurred owing to fading of permanganate.

Procedure

Reagents—

Redistilled water throughout.

Benzidine (*p*-diaminobiphenyl). A 1 per cent solution in 5 per cent acetic acid, stored in a dark bottle. The benzidine was purified (10) as follows: "20 gm. of benzidine were dissolved in 150 cc. of 70 per cent alcohol (7 volumes of 95 per cent alcohol plus 3 volumes of water) at about 70°, allowed to crystallize in the refrigerator, filtered with suction, and washed with a little cold 50 per cent alcohol. The crystals were redissolved in about 135 cc. of 70 per cent alcohol." 1 gm. of carbon was added and after further heating, filtered hot on a hot water funnel through paper previously washed with some hot alcohol. "Finally the flask and filter were washed with about 30 cc. of warm 50 per cent alcohol. The filtrate was cooled in the refrigerator, and the benzidine filtered with suction, and dried in a dark place." The air-dried substance contained water and traces of carbon which were removed by recrystallization from dry benzene. The product was stored in a bottle of brown glass.

Nitric acid (concentrated) free from all traces of oxides of nitrogen.

Sodium bismuthate, NaBiO_3 .

Shredded asbestos, purified by boiling with HNO_3 and washing thoroughly with redistilled water.

Standard manganese solution containing 1 microgram per cc., made by proper dilution of a solution containing 0.1 mg. per cc.

Method

A sample containing approximately 1 to 10 micrograms of manganese is weighed out and ashed in a well extracted porcelain dish at cherry-red heat. The ash is taken up in 3 to 5 cc. of concentrated HNO_3 and 10 cc. of water. The mixture is heated to boiling to permit thorough extraction of the ash and transferred quantitatively to a 100 cc. beaker. It is then evaporated to dry-

ness on a hot-plate, again taken up in 3 to 5 cc. of concentrated HNO_3 and 10 cc. of water, and again evaporated to dryness. This procedure is carried out a third time. The sample, which should now be free of chlorides, is taken up in 3 cc. of concentrated HNO_3 and 10 cc. of water and warmed slightly to bring into solution. Allow to cool and add approximately 0.2 gm. of sodium bismuthate; heat and allow to boil for 2 to 3 minutes. Cool to below 30° (11), add 0.3 to 0.5 gm. of sodium bismuthate, mix thoroughly, and let stand a few minutes to allow the excess sodium bismuthate to decompose. The excess sodium bismuthate is filtered off through a Gooch crucible. The Gooch crucible and suction flask are so arranged that the solution filters directly into the colorimeter tube containing 2 drops of the benzidine solution in 3 cc. of redistilled water. The solution is made up to a volume of 25 cc. (the colorimeter tubes having been previously calibrated to this volume) and thoroughly mixed. A yellow-green color develops which is read at the end of 5 minutes on the Evelyn photoelectric colorimeter with a 4200 Å. filter. Blanks prepared by using the same amounts of nitric acid and sodium bismuthate should be water-white.

It has been found possible to perform six to eight filtrations through the same filter and then read the colors developed on the colorimeter. In this way thirty to forty determinations can be read in 1 hour.

Results

Known solutions of manganese were analyzed; the amounts of manganese found are given in Table II. The best results are obtained when the sample contains from 1 to 10 micrograms of manganese.

Several samples of food mixtures were analyzed by this procedure and that described by Skinner and Peterson (12). The results obtained are as follows:

Sample No.	Method	No. of determinations	Mn found per gm. micrograms
R604	Periodate	2	3.35
R604	Benzidine	6	3.36 (3.2-3.6)
R610	Periodate	2	3.8
R610	Benzidine	3	3.92

TABLE II
Analysis of Known Solutions for Manganese Content

Mn taken	Mn found
micrograms	micrograms
0.1	0.13
0.25	0.21
0.5	0.61
1.0	1.2
2.0	2.0
4.0	4.1
5.0	5.1
7.0	7.0
10.0	9.95
12.0	12.3
15.0	15.4

TABLE III
Recoveries Obtained with Added Manganese

Sample	Weight of sample	Mn in sample	Mn added	Mn recovered	Added Mn recovered
	gm.	micrograms	micrograms	micrograms	per cent
604	5	39.7	20	20.1	101
604	5	38.0	20	19.5	97.5
610	5	39.8	20	20.3	102
610	1	7.11	3	3.2	106
CaCO ₃	1	112.0	50	49	98
Ca ₃ (PO ₄) ₂	0.5	29.5	5	5	100
NaCl	5	8.9	5	4.6	92
Calcium lactate	0.5	10.7	5	4.65	93

TABLE IV
Analysis of Bone for Manganese Content

Ration	Weight of bone	Mn found	Mn per gm. bone
	gm.	micrograms	micrograms
Low Mn	2.40	5.62	2.34
" "	1.65	3.15	1.92
" "	0.54	0.76	1.42
" "	1.50	2.94	1.96
" "	1.42	3.34	2.35
" "	1.52	3.63	2.39
Mn added	0.97	3.63	3.74
" "	1.88	10.70	5.70
" "	1.68	6.79	4.04
" "	1.42	8.94	6.31
" "	1.86	8.45	4.54
" "	2.07	7.46	3.60

Known amounts of manganese were added to the various samples; the recoveries obtained are shown in Table III.

Individual bone samples of chickens were analyzed for their manganese content. One group received a low manganese ration; the other the same ration plus 50 parts per million of added manganese. The results obtained are shown in Table IV.

Preliminary determinations were made on the blood of chickens receiving the same rations as above, the results of which are as follows:

Ration	Weight of blood	Mn found	Mn per 100 gm. blood
	<i>gm.</i>	<i>micrograms</i>	<i>micrograms</i>
Low Mn	44	3.4	8.00
Added Mn	37	5.95	16.00

In all our work the Evelyn photoelectric colorimeter was used. Since some laboratories may not be equipped with this instrument, the applicability of the method was studied with the Dubosq colorimeter. It was found possible to determine between 2 and 15 micrograms of manganese with this instrument. This procedure is neither as sensitive nor as rapid as with the use of the Evelyn photoelectric colorimeter.

SUMMARY

A method for the determination of 0.1 to 10 micrograms of manganese with the Evelyn photoelectric colorimeter has been described. Recoveries ranging from 92 to 106 per cent have been obtained.

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RADIOACTIVE PHOSPHORUS AS AN INDICATOR OF PHOSPHOLIPID METABOLISM*

V. ON THE MECHANISM OF THE ACTION OF CHOLINE UPON THE LIVER OF THE FAT-FED RAT

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One of the curious findings in lipid metabolism is the action of choline on fatty livers. Hershey (1) first showed that lecithin had a striking effect upon the liver of the depancreatized dog maintained with insulin, but it remained for Best and his coworkers (2, 3) to demonstrate that its action in reducing the fat content in the liver in this preparation as well as in the rat fed a high fat diet was due to its choline content. The curative and preventive action of choline upon the fatty liver of the depancreatized dog receiving insulin has been confirmed by Kaplan and Chaikoff (4), who have shown that its action in this respect is a slow one and that the daily feeding of large amounts for long periods is required to produce measurable changes in the lipid content of the liver of the depancreatized dog.

Despite the vast amount of work that has been done with choline, no *direct* experimental evidence has so far been provided to explain its action upon lipid metabolism, although a number of interesting speculations have appeared. The results of the present investigation indicate that choline definitely accelerates the synthesis and transfer of phospholipid by the liver of the fat-fed rat. This step in the intermediary metabolism of fat has been revealed with the aid of radioactive phosphorus, which has been previously used in this laboratory for identifying the rates of phospholipid turnover in various tissues of the intact animal

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(5-8). This study again shows the values of the radioactive indicator in phospholipid metabolism, for the acceleration in phospholipid metabolism produced by choline, although definite enough when the phospholipid molecule is radioactively labeled, is of such a small order of magnitude as compared with the total phospholipid content of the tissue and lasts for so short a time that its detection by measuring the total phospholipid content of the liver would be extremely problematical.

EXPERIMENTAL

The results recorded in this study were obtained from individual analyses of 250 rats, the data of 160 of which are shown below. The high fat, low protein diet employed consisted of 40 per cent butter fat, 50 per cent glucose, 5 per cent extracted casein,¹ and 5 per cent salt mixture (9). Vitamin supplements were either added to the diet or fed individually to the rats. The vitamin B complex² was furnished as a concentrate obtained from rice bran, which was supplemented with a clay rich in adsorbed vitamin G. A standardized cod liver oil supplied vitamins A and D. Rats were maintained on the diet for periods varying from 3 days to 3 weeks before the administration of choline and radioactive phosphorus.

The animals were permitted access to food during the entire period of observation, which was not interrupted before or after the choline feeding. Radioactive phosphorus was administered subcutaneously as an isotonic solution of Na_2HPO_4 . Choline in the form of its chloride was fed by stomach tube.

The livers and, in some cases, the small intestines were removed at various intervals after choline or phosphorus administration. Phospholipids were isolated from these tissues in the manner previously described (5). The method by which the phospholipids

¹ The casein used in this study was extracted for several days with increasing concentrations of methanol, the final extraction being with 90 per cent hot methanol for 1 day.

² Each rat received approximately 0.10 cc. of the vitamin B concentrate per day. This concentrate contained about 50 i. u. of vitamin B₁ per cc. and 10 modified Bourquin-Sherman units of G (flavin) per cc. The same concentrate has also been shown to be a good source of both rat and chick antidermatitis factors (10).

were mounted and their radioactivity determined has also been noted elsewhere (5).

Results

Action of Choline in Rats Fed a High Fat, Low Protein Diet for 3 Weeks—Twenty-four female rats were used in this experiment. After they had been maintained on this diet for 14 days, they were treated as follows: (A) Six rats received 25 mg. of choline chloride daily for 4 days. 3 mg. of labeled phosphorus in the form of an isotonic solution of Na_2HPO_4 were injected subcutaneously at the same time that the last dose of choline was given. The livers of the animals were removed for analyses at 6 and 16 hours after the last administration of phosphorus and choline. (B) Six rats were fed 25 mg. of choline chloride daily for 8 days, while the injection of 3 mg. of labeled phosphorus in the same form as above was made at the time of the last choline feeding. The livers were removed at 6 and 16 hours after the phosphorus treatment. (C) Twelve rats were used for controls, six for Group A and six for Group B. These received no choline, but the labeled phosphorus was injected subcutaneously in amounts equal to and at the same time as that received by their respective groups.

The livers of these rats contained from 6.4 to 20.6 per cent total lipids (average 12.4 per cent) at the time they were examined for phospholipid activity.

The amounts of labeled phospholipid found in the whole livers³ of control and choline-treated rats at two intervals (6 to 16 hours) after the administration of radioactive phosphorus are shown in Table I. At the 6 hour interval the content of labeled phospholipid in the livers of the choline-treated rats was definitely above that found in the control animals. This was true for the rats that received 25 mg. of choline chloride daily for 8 days as well as for those that received this amount daily for 4 days. Thus the mean values for the percentage of the administered phosphorus incorporated into liver phospholipid at the 6 hour interval were

³ In the present study the activities of whole livers instead of per gm. of tissue were compared. Animals were carefully paired according to weight and sex.

2.36 and 2.12 for Groups A and B respectively, whereas similar mean conversion values found in the livers of control animals were 1.63 and 1.48. Table I shows that the effect of choline is more pronounced 6 hours after being given than at 16 hours. At the latter period the differences between the choline-fed animals and controls may hardly be considered significant.

TABLE I

Effect of Choline on Phospholipid Metabolism of Fatty Livers

The rats had received a high fat, low protein diet for 18 to 22 days at the time the livers were analyzed.

Activity refers to per cent of administered labeled phosphorus found as phospholipid.

Group A Choline-treated for 4 days			Control		Group B Choline-treated for 8 days			Control	
Time killed after choline feeding	Rat No.	Activity	Rat No.	Activity	Time killed after choline feeding	Rat No.	Activity	Rat No.	Activity
<i>hrs.</i>					<i>hrs.</i>				
6	85	2.12	97	1.70	6	91	1.94	103	1.31
	86	2.67	98	1.71		92	2.23	104	1.79
	87	2.32	99	1.49		93	2.20	105	1.33
Average.....		2.36		1.63			2.12		1.48
% increase....	45						43		
16	88	1.89	100	1.73	16	94	1.49	106	1.45
	89	2.04	101	1.49		95	1.57	107	1.50
	90	2.06	102	1.85		96	1.37	108	1.18
Average.....		1.98		1.68			1.48		1.36
% increase....	18						9		

Effect of Single Administration of Choline in Rats Fed a High Fat, Low Protein Diet for 3 Days—In view of the above finding that four daily administrations of choline were as effective as eight, it was deemed advisable to study the effect of a single feeding of this substance upon phospholipid turnover. Sixteen female and eight male rats were fed the high fat, low protein diet for 3 days, and on the 4th day half of each group was given 3 mg. of labeled phosphorus and 30 mg. of choline chloride simultaneously.

The remaining twelve rats were given only the phosphorus. All twenty-four animals were killed 4 hours after the phosphorus administration. The results obtained from the radioactive phospholipid content, stated as a percentage of the administered labeled phosphorus, are shown in Table II. The average values for control rats were 2.15 and 2.31 per cent for female and male groups respectively. The corresponding choline-treated rats

TABLE II

Effect of Choline on Rats Fed Fat for Short Intervals

Activity refers to per cent of administered labeled phosphorus found as phospholipid.

	Controls		Choline-treated	
	Rat No.	Activity	Rat No.	Activity
Females	273	2.12	265	3.04
	274	2.05	266	2.58
	275	2.25	267	2.90
	276	2.27	268	2.61
	277	2.41	269	2.85
	278	1.84	270	2.50
	279	2.19	271	2.53
	280	2.07	272	2.68
Average.....		2.15		2.71
% increase.....				26
Males	291	2.01	281	2.92
	292	2.30	285	3.04
	295	2.52	286	3.38
	296	2.41	287	3.18
Average.....		2.31		3.13
% increase.....				35

showed average values of 2.70 and 3.16 per cent. Thus the differences between the livers of treated and untreated rats are comparable with the values observed at the 6 hour interval in Table I.

Duration of Action of Choline—Two separate series, each containing forty female rats, were maintained on the high fat, low protein diet for 14 days, at the end of which twenty of each

group were fed 25 mg. of choline chloride daily for 3 successive days. On the 3rd day 3 mg. of labeled phosphorus were injected at the same time that the last choline feeding was made. Thereafter rats were killed for liver examination at the following intervals after the choline administration: 1, 3, 6, 12, and 24 hours. The results are shown in Fig. 1 on which each point represents the average value obtained from eight separate analyses for individual animals. The curves show in a striking manner that the action of choline in the acceleration of phospholipid formation in the liver takes place over a limited interval of time after choline

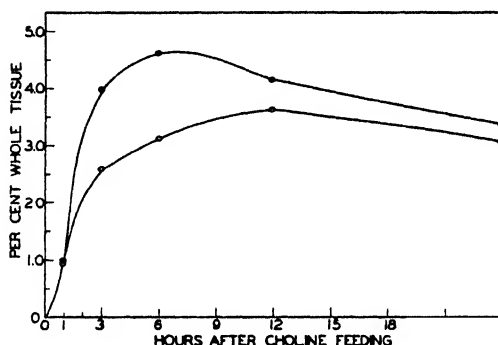


FIG. 1. The duration of the action of a single dose of choline upon the phospholipid metabolism of the liver. The ordinates represent the per cent of administered labeled phosphorus in the phospholipid of the whole liver. ●, obtained from rats fed choline chloride; ○, from rats fed no choline chloride. Each point represents the average of eight separate liver analyses.

administration. Practically no effect was demonstrated before 1 hour. The maximum effect was found between 3 and 8 hours after the choline feeding, while at the 12 hour interval the phospholipid activity of the choline-treated rats approached that of the controls.

In one series of forty rats the small intestines were also examined for phospholipid activity. Although the choline-treated animals maintained a higher phospholipid activity for several hours after the choline feeding, the differences from the controls are not striking.

Effect of Varying Choline Dosage upon Phospholipid Turnover—

Thirty-two rats were fed the high fat, low protein diet for 3 days and then treated as follows: all rats were injected with 3 mg. of labeled phosphorus but their choline treatments differed; eight rats received no choline but the other three groups of eight rats each received 2, 10, and 30 mg. of choline chloride respectively; as in previous experiments, choline and phosphorus were administered simultaneously, and all animals were killed 4 hours later. The results are shown in Fig. 2. A definite relation between the amount of choline fed and the level of newly formed phospholipid is brought out by these data. Thus, under the influence of 30 mg. of choline chloride, the livers obtained from

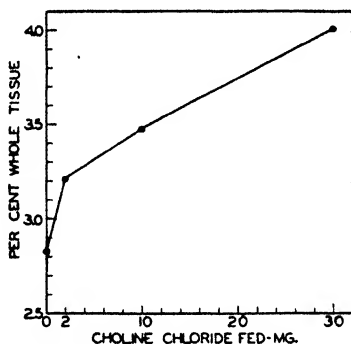


FIG. 2. The effect of varying choline dosage upon phospholipid turnover in the liver. The ordinates represent the per cent of administered labeled phosphorus in the phospholipid of the whole liver. Each point represents the average of eight separate liver analyses.

eight rats showed an average phospholipid activity of 4.0 per cent as compared with 3.5 for rats receiving 10 mg. each, 3.2 for those fed 2 mg. per rat, and 2.8 for those that received no choline.

DISCUSSION

As previously pointed out, the formation and destruction of phospholipid can be followed within the animal by means of a radioactive isotope of phosphorus (5-8). After entering, the labeled phosphorus becomes intimately mixed with similar unlabeled molecules, but since the ratio of labeled to unlabeled molecules would be difficult to obtain, it has not been found possible in this study to determine the total amount of newly

synthesized phospholipid; *i.e.*, the quantity formed from unlabeled plus labeled phosphorus. The values obtained for the synthesized *labeled* phospholipid during any interval, however, are directly proportional to the *total* amount formed during this period; hence for comparative studies (as regards different tissues within the same animal or the same tissue in different groups of animals) a measure of the labeled phospholipid synthesized is just as significant as the total phospholipid. Thus the higher labeled phospholipid values found in the livers of the choline-treated rats as compared with the untreated animals show that more rapid synthesis and transfer of phospholipid are occurring in the former than in the latter.

Although the difference between the labeled phospholipid content of the treated and untreated livers does not exceed 40 per cent at the height of the choline action, this difference nevertheless assumes considerable significance when it is recognized that phospholipid metabolism is a dynamic process in which the newly formed phospholipid is rapidly removed. The rapidity of the synthesis as well as of the removal of phospholipid from the liver has been stressed in previous reports from this laboratory in which it was shown that even in the fasted state the liver carries on a rapid phospholipid turnover (5).

A point of fundamental importance is the relatively short interval during which the effects of a single administration of choline are demonstrable. It was found that a change in phospholipid activity begins approximately 1 hour after choline ingestion and that its effect is greatly diminished or altogether absent about 10 hours later. The effect of choline upon phospholipid metabolism also depends upon dosage, for it was found that between the limits of 0 and 30 mg. of choline chloride there was a relation between the amount fed and the phospholipid response (Fig. 2). This provides further evidence for the specific action of choline upon phospholipid metabolism.

Several mechanisms have been suggested to explain the actions of choline upon fatty livers. Best, Channon, and Ridout (11) consider it most probable that choline speeds up the oxidation of fatty acids by the liver itself. Others have attempted to show the presence of choline derivatives in liver phospholipid after their ingestion. Thus Welch (12) fed an arsenocholine and showed its presence in the liver phospholipid, whereas Channon

et al. (13), who fed a triethylcholine, failed to identify this in the phospholipid isolated from the liver. Such studies, however, cannot shed light on the mechanism of the action of choline, for at most they can show that dietary choline enters into phospholipid formation. There is no reason to believe that the organism distinguishes between exogenous and endogenous choline every time a new phospholipid molecule is being formed. The inclusion of fed choline or one of its derivatives in the phospholipid deposited in the liver, while of interest to anyone studying their fate in the body, cannot yield information on the rate of phospholipid turnover by the liver, much less show comparative rates before and after choline treatments.

The fate of two substances remains to be considered: the choline administered and the additional phospholipid formed under the influence of choline. In regard to the former, it should be stressed that, while choline stimulates phospholipid metabolism, it does not necessarily follow from the evidence provided here that the increased amount of phospholipid synthesized by the liver occurs at the expense of the fed choline. Moreover, while it would not be profitable to speculate, with our present knowledge, upon the ultimate fate of the increased amount of labeled phospholipid formed under the influence of choline, it has nevertheless been shown above that it does not remain in the liver for long. It is either broken down (utilized) in the liver or transferred to some other tissue.

We are indebted to Professor E. O. Lawrence and members of the Radiation Laboratory for the preparation of the radioactive phosphorus that made this study possible. Assistance was also furnished by the Works Progress Administration.

SUMMARY

1. The rate of phospholipid turnover was compared in the livers of choline-treated and untreated rats with the aid of a radioactive isotope of phosphorus.

2. It is shown that choline speeds up phospholipid metabolism in the liver. An increased formation as well as a rapid removal of phospholipid was found in the livers of rats after the ingestion of choline.

3. The time during which a single administration of choline

speeds up the phospholipid metabolism has been defined. An increased phospholipid metabolism appears approximately 1 hour after choline ingestion and its effect has disappeared by about 10 to 12 hours thereafter.

4. The increase in phospholipid formation is proportional to the amount of ingested choline (between the doses of 0 and 30 mg. of choline chloride per rat).

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SPECIFIC AND NON-SPECIFIC CELL POLYSACCHARIDES OF A BOVINE STRAIN OF TUBERCLE BACILLUS*

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In previous communications (1-3) the writers have reported on the complex mixture of polysaccharides extracted from defatted cells of a human strain, H-37, of the tubercle bacillus. The cell polysaccharides of bovine strain, M-1698,¹ have now been fractionated in similar fashion by methods in which the use of alkali, mineral acid, and heat is avoided. As difficulties were occasioned by the presence of large amounts of polysaccharides which did not react with available sera, details are given for modifications of the earlier procedure.

EXPERIMENTAL

570 gm. of dried, defatted, and ground bovine bacilli, Strain M-1698, were percolated with dilute acetic acid containing 0.5 per cent of phenol. After about 3 months 72 liters of percolate were collected; extraction was then continued for about 1½ years, during which an additional 55 liters of percolate were obtained. The two portions were worked up separately as Lots 530 and 531. As in the human type preparation (3) all precipitations were repeated until material of lower and higher solubilities than the

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† National Tuberculosis Association Fellow.

¹ We are indebted to the Mulford Biological Laboratories of Sharp and Dohme, Glenolden, Pennsylvania, Dr. John Reichel, Director, for the tubercle bacilli and anti-human, bovine, and timothy strain horse sera used.

fraction taken was eliminated. The by-products obtained in this way were added to the appropriate fractions.

Many of the fractions of Lot 530 contained strongly colored impurities, removal of which was often quite difficult. The following procedure was usually found effective:² 2 gm. or less of the carbohydrate were dissolved in 3 ml. of water and 1 ml. of saturated copper acetate solution was added, followed by methyl alcohol in 1 ml. portions until flocculation began. After addition of 1 ml. more of methyl alcohol and short standing the suspension was centrifuged; the supernatant fluid and two washings with 60 per cent methyl alcohol were poured either into an excess of glacial acetic acid or into acetone containing glacial acetic acid and LiCl. This precipitated the carbohydrate and left most of the Cu⁺⁺ in solution. Residual copper was removed by solution in a little water, addition of 3 volumes of glacial acetic acid containing 2 per cent LiCl, followed by addition of 30 volumes of acetone. This process was repeated until the product contained less than 0.004 mg. of copper, as shown by the absence of yellow color in the supernatant fluid. The copper-precipitable portions from Fractions B₁, B₂, and B₃ (Flow Sheet III) were combined, freed from copper, and isolated as Fraction B₄ (Table I).

Lot 530

The separation of the principal crude fractions, C, B₂, and B₁, corresponding to those obtained from the human strain (3), is indicated in Flow Sheet I.

Fraction 530 C—Unlike the corresponding human type carbohydrate, this product not only gave a portion, C₁, precipitable at 80 per cent acetic acid concentration, but also contained large amounts of more soluble material, precipitable at higher concentrations of acetic acid (Flow Sheet II).

Fractions 530 B₁ and B₂—The treatment given these crude portions of the bovine carbohydrate mixture is indicated in Flow Sheet III.

The yields and properties of the various final fractions are included in Table I.

Carbohydrate Fractions Soluble in Strong Acetic Acid (Flow

² For another modification of this procedure and isolation of a pigment see below.

TABLE I

Properties of Final Polysaccharide Fractions of Bovine Strain M-1698 of Tubercle Bacillus

Precipitin tests after centrifugation are given in parentheses.

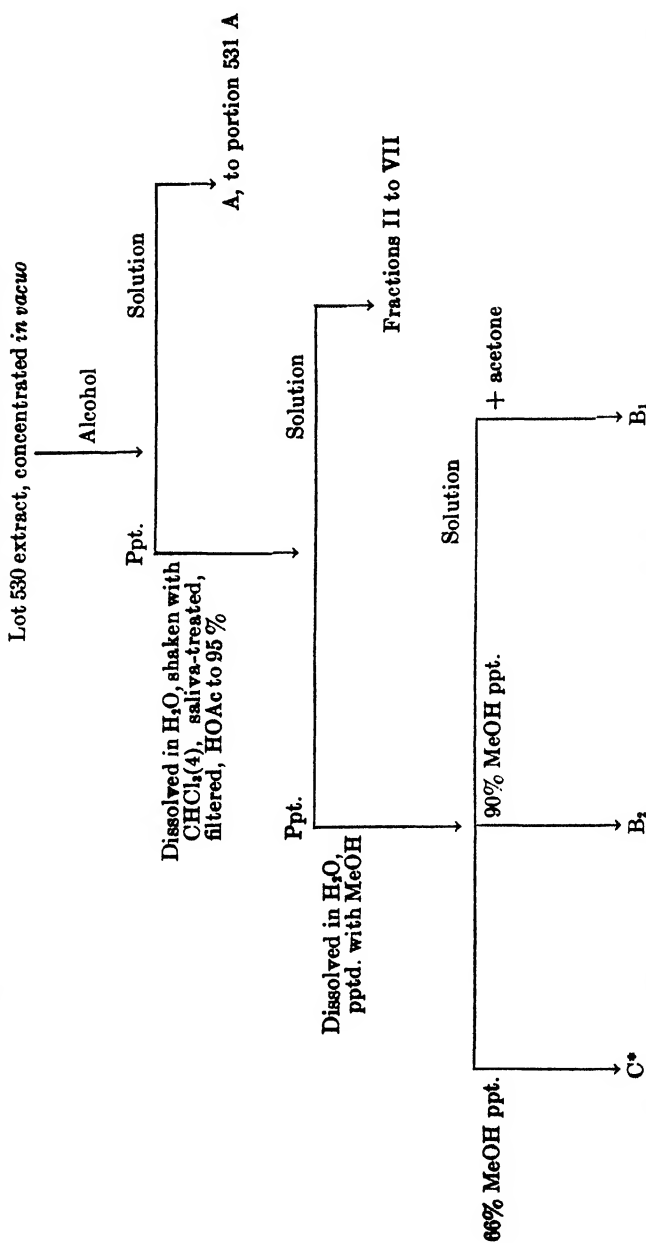
Fraction No.	Yield	$[\alpha]_D$	$[\alpha]_{545}$	Neutral equiv- alent	N	P	Pentose	Ino- site	Pptn. at 1:500,000 dilution with Serum 5807-L
	gm.	degrees	degrees		per cent	per cent			
530 C ₁	7.0								
C _{1a} *	(0.14)	+83	+104	800					-(+)
C _{1b} *	(5.6)	+92	+108	1500	0.1	5.9	++	-	-(-)
C _{2a}	0.01	+107	+131	1200		4.6			±(+±)
C _{2b}	0.40	+86	+102	1300	0.7	5.0	+++	±	±(+)
C _{2c}	1.72	+96	+114	4500	0.2	5.1	+++	-	-(+±)
C _{2d}	0.20	+92	+111	2500	0.7	4.3	++±		-(+±)
C _{2d'}	0.07	+95	+114	1400	0.7	7.0	±	+	-(-)
C _{2e}	0.98	+91	+111	1200	0.4	4.8	+	-	±(+±)
C _{2f}	0.52	+97	+115	1500	0.6	6.1	±	-	-(±)
C _{3a}	1.49	+96	+115	1200	0.6	6.3	++	-	-(-?)
C _{3b}	0.45	+98	+117	1200	0.6	6.1	+±	-	-(-)
C _{3c}	0.15	+98	+118	1100	1.1	6.4	±	-	-(-)
C _{4a}	0.66	+91	+110	1500	0.9	6.1	+	+	-(-)
C _{4b}	1.86	+97	+115	3100	0.0	6.1	-	-	-(-)
B ₂	0.49	+94	+106	3300	0.4	3.7	+++	-	±(+±)
B ₂	0.72	+87	+101	5700	0.2	2.1	+++	-	±(+±)
B _{2'}	0.40	+92	+110	2500	1.0	4.8	±	+	-(-)
B ₁	0.70	+51	+63	5000	0.2	2.6	++	-	+(++)
B ₂ C	3.48	+92	+108	1000	0.2	6.3	++	-	-(-)
B ₂ C	3.28	+90	+107	1000	0.2	6.1	-	-	-(-)
B _{2'} C	0.14	+96	+116	2500	0.3	6.0	-	±	-(-)
B ₁ C	0.32	+102	+123	1600	0.0	5.7	±	-	-(±?)
B ₄	0.12	+62			2.6	4.6	+++	±	-(+±)
531 C ₁	2.22	+85	+101	2100	0.1	1.8	++	-	+±
C ₂	0.29	+74	+86	2300	0.8	2.4	+±	-	+(+±)
C ₃	0.04	+54	+64	900	4.2	2.8			±(+)
B ₂ †	0.37	+65		2600	1.5	1.4	+++±	±	+±
B _{2a}	0.18	+58	+68	3600	0.4	2.2	++	-	+±
B ₁	0.40	+21	+25	4200	0.3	1.3	+++	-	+(++)
B ₂ C	1.73	+89	+106	1900	0.2	2.1	++±	-	-(+±)
B ₂ C _a	0.43	+57	+69	2600	0.3	2.2	++++	-	+(++)
B ₁ C	0.42	-8	-11	5300	0.1	1.3	++	-	+(++)

* Only an aliquot portion was fractionated.

† Basic ash, as Ca, 0.8 per cent.

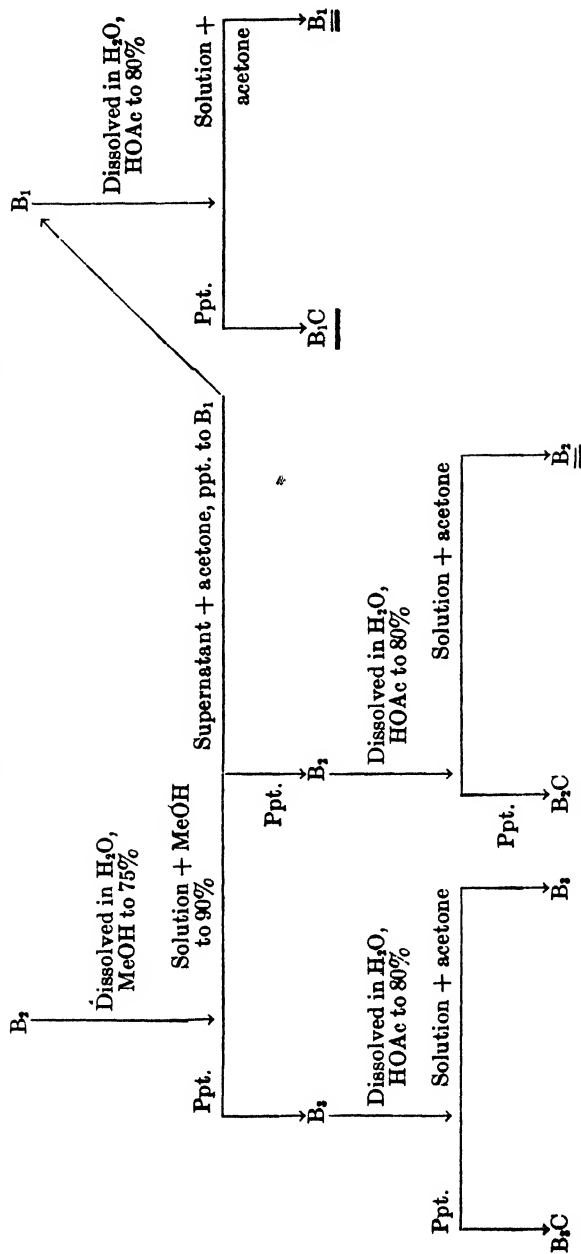
Flow Sheet I

Separation of Three Main Fractions of Carbohydrates from Bovine Tubercle Bacillus Cells



* In unsuccessful attempts to remove colored impurities a small serologically inactive fraction, B₂', was found soluble in 62.5 per cent EtOH.

Flow Sheet III
Separation of Fractions B₂ and B₁ (Flow Sheet I) into Component Portions



The underlined fractions showed relatively high serological activity, a light line indicating Lot 530, a heavy line indicating Lot 531.

Sheet I)—Inorganic phosphates were removed with uranyl acetate. As all of the fractions from this material were serologically inactive or only slightly active in the antisera available, details of their isolation are omitted. The designations (Table II)

TABLE II

Properties of Bovine Tubercle Bacillus Carbohydrate Fractions Soluble in Strong Acetic Acid

Only Fractions 531 IIb and IVa gave positive precipitin reactions at 1:500,000 dilution and higher. The parentheses indicate precipitation after centrifugation.

Fraction No.	Yield	$[\alpha]_D$	$[\alpha]_{488}$	Neutral equivalent	N	P	Pentose	Ino-site	Pptn. at 1:50,000 dilution with Serum 5807-L
	gm.	degrees	degrees		per cent	per cent			
530 IIa	0.98	+62	+76	800	0.7	3.8	±	+	-(±)
IIb	2.20	+90	+108	1600	0.6	4.5	-	-	±(+±)
IIc	0.09	+76	+92	1900	1.3	2.0	-	±	+(++)
IIIa	11.22	+54	+60	1500	1.5	2.6	-	-	-(±)
IIIb	2.74	+58	+73	1500	1.9	2.3	+	-	-(+±)
IIIc	0.53	+70	+79	1300	2.8	2.0	±	-	-(±)
IVa	2.28	+40	+48	2700	2.2	1.0	±	-	-(-)
IVb	0.18	+19	+24	4500	1.9	0.5	-	-	-(-)
V	5.49	+15	+19	2200	2.9	0.6	-	-	-(-)
VI	4.32	+2	+3	5500	0.9	Trace	-	-	-(-)
VII	0.10	+3		1000	3.4	"	++		-(-)
531 II	0.09	+77	+90	600	0.7	4.7	+	-	+(+±)
IIIa	1.63	+54	+65	3400	2.2	2.8	+	+	+±
IIIb	0.54	+40	+49	4200	3.4	2.1	+++	±	++
IVa	0.33	+27	+33	1600	2.9	1.3	+++	-	++±
IVb	1.11	+16	+18	1200	7.5	1.1	-	-	+(+±)
V	0.10	-11	-16	2500	8.2	0.8	+±	±	+(+±)
VI*	2.81	+3	+4	3100	1.6	Trace	-	-	-(±)
VIa	0.14	+1	+1	Very high	0.2	None	-	-	-(-)

* Basic ash, as Ca, 19.1 per cent.

correspond roughly to those of analogous products from the human strain except that Fraction VI was soluble in absolute methyl alcohol (with precipitation of accompanying salts) and was precipitated with acetone.

Lot 531

This material, from the 55 liters collected after the first percolation, was handled according to the same general scheme as Lot 530. Unless otherwise stated, corresponding fractions bear the same designations. A portion soluble in about 10 volumes of alcohol (Fraction 531 A) was combined with Fraction 530 A (Flow Sheet I).

Fractions Precipitable with Strong Acetic Acid—The alcohol-precipitable part was divided into a portion precipitable at an acetic acid concentration of about 97 per cent and a portion soluble at this concentration. The former material was separated, much as in Flow Sheet I, into Fractions C, B₂, and B₁. Fraction C was resolved as in Lot 530 (Flow Sheet II) into C₁, C₂, and C₃; a C₄ fraction was not obtained. Fraction B₂ was separated into the final B₂ and B_{2a}, both soluble in 80 per cent acetic acid, the former precipitable, the latter soluble, at 90 per cent methyl alcohol concentration. The 80 per cent acetic acid precipitate yielded Fractions B₂C and B₂C_a, the former precipitable, the latter soluble, at 75 per cent methyl alcohol concentration. From Fraction B₁ no portion insoluble in 80 per cent acetic acid was obtained; Fraction B₁C was precipitable at 87.5 per cent acetic acid and B₁ was precipitated from the supernatant by excess acetone. Copper acetate treatment was unnecessary, as was also the extensive subfractionation carried out on Lot 530 because of excessive amounts of serologically inactive components. The yields and properties of these fractions are recorded in Table I.

Carbohydrates Soluble in Strong Acetic Acid—The separation of these fractions, which correspond to Fractions II to VII of Flow Sheet I, was also effected much as for Lot 530. Fraction VI was in this instance precipitated from absolute alcoholic solution with a moderate amount of acetone, while Fraction VIa resulted from the addition of much acetone. The yields and properties of these fractions are recorded in Table II. The supernatants of Fraction VIa yielded only a small amount of ether-soluble oil.

Fractions from Combined Solutions, 530 A and 531 A (Flow Sheet I)

Both alcoholic solutions were highly colored and contained serologically active carbohydrates, which were further fractionated with alcohol after concentration *in vacuo* to about 20 ml. A

portion, Fraction A_1 , was precipitated with about 4 volumes of alcohol. The brown supernatant was treated with a freshly prepared mixture of equal parts of saturated aqueous and alcoholic copper acetate solutions until no further flocculation occurred; a total of 25 ml. was necessary. The precipitate, Fraction A_2 , was centrifuged off and the supernatant, Fraction A_3 , was freed from copper with $K_4Fe(CN)_6$. Fraction A_2 was suspended in about 30 ml. of 1.5 N acetic acid and dialyzed against water, washed with water and acetone, and dried *in vacuo*. This product appears to be one of the pigments of the bovine tubercle bacillus. After isolation it is insoluble in water and common organic liquids but is somewhat soluble in dilute acetic and sulfuric acids and in 5 per cent NaOH. It dissolves with a deep brown color in 50 per cent H_2SO_4 and in strong NaOH; the solutions give negative Molisch and biuret tests. The pigment contained 18 per cent of nitrogen (micro-Kjeldahl) and 0.3 per cent of phosphorus; spectrophotometric examination showed no selective absorption.

Fraction A_1 in 50 per cent alcohol also yielded some of the copper-precipitable pigment, Fraction A_2 . Fractions A_1 and A_3 gave portions precipitable with alcohol at 80 per cent concentration (Fractions A_{1a} and A_3). Fractions soluble at this concentration but precipitable at about 95 per cent alcohol concentration were combined as Fraction A_{1b} . The supernatants from A_{1b} gave a fraction, A_4 , on addition of 3 volumes of acetone. The supernatant from this, concentrated *in vacuo* to dryness, taken up in methyl alcohol, and precipitated with about 10 volumes of acetone in the presence of LiCl, yielded Fraction A_5 . The supernatant from this was evaporated, taken up in 20 ml. of absolute ethyl alcohol, and precipitated with 150 ml. of absolute ether; the supernatant from this, Fraction A_6 , yielded a residue, Fraction A_7 .

The outside liquids from the dialyses contained extremely small amounts of material which were discarded.

Chemical Properties

The yields and properties of the fractions are summarized in Tables I to III. Basic ash was seldom determined owing to the presence of phosphorus. Fractions 530 IIa and 531 II contained about 0.3 per cent of inorganic phosphate; this was absent or

present only in traces in the other fractions. Inosite was found in some of the fractions by the Scherer test. Pentoses were roughly estimated by a modification of the Neumann test (5).

Fraction 531 C₁ was not markedly opalescent, as was the corresponding human fraction, 530 C, which yielded magnesium palmitate after treatment with alkali. However, the bovine fraction became turbid after several hours in alkaline solution and also deposited a white substance which was not further investigated.

TABLE III

Properties of Bovine Tubercle Bacillus Fractions Originally Soluble in Strong Alcohol

Only Fraction A_{1a} gave precipitin reactions at dilutions of 1:500,000 and higher.

Fraction No.	Yield	[α] _D	[α] ₅₈₉	Neutral equiv- alent	N	P	Pentose	Ino- site	Precipitin test at 1:50,000 dilution with Serum 5807-L
	gm.	degrees	degrees		per cent	per cent			
530-531 A _{1a}	0.71	+49		15,000	1.8	2.0	+++	+	+++
A _{1b}	1.29	+25	+32	6,200	3.8	0.7	++	-	+±
A ₂ , pigment	0.12				18.0	0.3			
A ₂ *	2.63	+39	+51	5,100	3.2	0.9	+	-	+±
A ₄	1.15	+7			5.9	0.7	+±	-	±(+)
A ₅	6.31	-2		11,000	1.8	0.6	-	-	-(-)
A ₆	0.26	-7		2,000	6.9		++++		-(-)
A ₇	0.48	-2			9.0		+++†		-(-)

* Basic ash, as Ca, 7.7 per cent.

† +++++ at Line 35 of the spectroscopic scale.

Serological Properties of Principal Fractions

The qualitative serological tests were carried out as described previously (3). All of the principal fractions were tested with anti-H-37 strain horse serum No. 5807-L, anti-bovine strain rabbit sera, an anti-bovine strain goat serum,³ and an anti-bovine

³ Furnished by Dr. Kenneth C. Smithburn of The Rockefeller Institute for Medical Research.

strain horse serum. The bovine type antisera were, unfortunately, weak in anticarbohydrate. As no evidence of a bovine type-specific substance was encountered, details of these tests are omitted. In the anti-human strain horse serum No. 5807-L the principal active fractions gave positive tests at dilutions of 1:2 million and 1:4 million; to emphasize differences between active and inactive material the reactions are recorded only at 1:50,000 and 1:500,000 in Tables I to III. Heterologous reactions were also observed with an anti-timothy bacillus horse serum and an anti-avian S strain rabbit serum. Qualitative cross-reactions, as well as quantitative determinations with Fractions 530 C₂, 531 C₁, and, for comparison, the human strain polysaccharide Fraction 520 C, were conducted (Table IV) according to previous descriptions (3). For the analyses 3.0 ml. of serum were used in duplicate and all manipulations were carried out in the cold, the tubes being allowed to stand for 3 days after the addition of amounts of polysaccharide indicated by preliminary tests. The second absorption with the same polysaccharide, as well as the cross-absorptions with Fraction 520 C, was run on aliquots from the supernatant fluids; the nitrogen precipitated was calculated to the original serum volume.

DISCUSSION

It is evident from the experimental data that the carbohydrate extracted from the defatted cells of a bovine strain of the tubercle bacillus consists of an even more complex mixture of serologically active and inactive polysaccharides than was obtained from the H-37 human strain (3). Glycogen was again encountered (*cf.* (6, 3)), as well as far larger amounts of pigment and serologically inactive material than were extracted from the human type cells.

The qualitative and quantitative tests summarized in Table IV show that the serological specificities of the bovine fractions do not differ essentially from those of the polysaccharides of the human type cell. The same two independently specific polysaccharides are present, as is also material combining both specificities. Thus the anti-human strain (H-37) horse serum No. 5807-L, after absorption with Fraction 531 C₁, gave definite precipitin reactions with the bovine Fraction 531 B₁ and the corresponding human fraction No. 520 B_{2a}. The absorbed serum also precipitated the

bovine Fractions 531 B₂, 530 B₁, and 530 B₂, and the human Fraction 520 B_{2a}, in none of which it had been possible to separate the specificities characteristic of the B and C fractions. Nevertheless, the possibility of minor differences between the C fractions of human and bovine origin was indicated by the weak reaction given by this serum (absorbed with Fraction 531 C₁) with the human Fraction 520 C.⁴ Similar considerations apply to the

TABLE IV

Qualitative Cross-Reactions with Absorbed Anti-H-37 Horse Serum 5807-L and Quantitative Tests with 3.0 Ml. of Unabsorbed Serum

Fraction No.	Serum absorbed with		Amount of fraction used for each quantitative pptn.	N pptd. by first addition	N pptd. by second addition of same fraction	N pptd. by Fraction 520 C from aliquot after first absorption
	Fraction 531 C ₁ *	Fraction 531 B ₁				
			mg.	mg.	mg.	mg.
531 C ₁	-(-)	-(+±)	0.15	0.24	0.02†	0.02†
B ₁	±(++)	-(-)				
B ₁ C†	+(++)	-(-)				
B ₂	+(++)	-(+)				
530 C _{2a}	-(-)	-(+)	0.30	0.25	0.03†	0.04†
B ₁	+(+±)	-(-)				
B ₂	+(++)	-(+)				
Human type						
520 C	-(+)	-(+±)	0.15	0.28	0.02†	
B _{2a}	++	-(±)				
B _{2a}	+±(++±)	-(+±)				

* Similar results were obtained by absorption of Serum 5807-L with Fraction 530 C_{2a}.

† Calculated to 3.0 ml., the original volume.

‡ In one experiment this fraction absorbed all the anticarbohydrate from Serum 5807-L; in another experiment weak reactions remained with Fraction 531 C₁ and human bacillus Fractions 520 C and 520 B_{2a}.

independently specific B fractions, as shown by the results with Serum 5807-L absorbed with Fraction 531 B₁ (Table IV).

The principal differences between the human and bovine material lay in the distribution of the fractions carrying these specificities (Table V). About 53 per cent of the active poly-

⁴ Similar minor differences are also indicated by the quantitative data in Table IV.

saccharides isolated from the acetic acid-precipitable portions of the two bovine preparations were accounted for as C fractions (precipitable by methyl alcohol at about 66 per cent concentration) and only about 47 per cent as B fractions (soluble at 66 per cent methyl alcohol concentration). In the human preparation, on the other hand, the content of B fractions was about 7 times that of the C fractions.

Of the inactive substances isolated from the bovine cells, two were strongly dextrorotatory and exceedingly difficult to separate from the serologically active high rotating portion, Fraction C. Both contained organic phosphorus but differed greatly in their

TABLE V

*Proportion of Serologically Active and Inactive Polysaccharide Fractions
Extracted from Human and Bovine Type Tubercle Bacillus Cells*

Lot No.	Total yield		Total active material		Total inactive material		Proportion of active material in total carbohydrate
	gm.	per cent*	gm.	per cent*	gm.	per cent*	per cent
520 (human type).....	38	6.3	33	5.5	5	0.8	87
530 (bovine).....	56	9.8	6	1.0	50	8.8	11
531 (").....	13	2.3	7	1.2	6	1.1	54
530, 531 A.....	13	2.3	1	0.2	12	2.1	8
Total for bovine fractions.....	82	14.4	14	2.5	68	12.0	17

* Of defatted tubercle bacillus cells.

solubilities. One of these inactive substances, precipitable at 80 per cent acetic acid concentration, was the principal component of Fractions 530 B₂C, B₂C, B₂·C, and B₁C, and was analogous to the inactive material encountered in the human preparation (Fractions 520 B₂C and B₁C (3)). The other product, soluble at 96 per cent acetic acid concentration, was isolated as Fractions 530 C_{4a} and C_{4b}, and was found only in the bovine strain. If this phosphorylated carbohydrate occurs in all strains of bovine origin, and only in these, it would serve to characterize these strains chemically. Although most of the acetic acid-soluble fractions of the human preparation were serologically active, the corresponding fractions of bovine origin were generally in-

active. One of these inactive fractions, 530 VI, showed unusual solubility in methyl alcohol and absolute ethyl alcohol. This highly hygroscopic substance was almost optically inactive and contained no phosphorus or pentose. The Molisch test was positive only up to a dilution of 1:5000, while in the other acetic acid-soluble fractions the Molisch test was distinct up to a dilution of about 1:50,000. Fractions 531 VI and 530 A₅ showed similar behavior. These products, also, were not encountered among the human cell polysaccharides. It is conceivable that more potent antisera than were available for this investigation would contain antibodies to some of these fractions which appear to be serologically inactive but chemically specific for the bovine strain, as opposed to the human strain.

A compilation is given in Table V of the yields and distribution of active and inactive fractions isolated from the bovine and human type tubercle bacillus cells. While about 87 per cent of the polysaccharide fractions obtained from the human strain were found to be strongly active serologically, only 17 per cent of the total carbohydrate mixture of bovine origin showed serological activity sufficient to give a weak positive reaction with Serum 5807-L at a dilution of 1:500,000. The total extractable carbohydrate content of the bovine type cells was more than twice that of the human strain cells. In the bovine preparation most of the inactive material accumulated in the first percolate (Lot 530), but even in the second percolate (Lot 531) the active material did not approach the high percentage in the human type extract. The acetic acid-precipitable portion of Lot 531 yielded only active fractions (*cf.* Table I). Even Fractions 531 B₂C and B₁C were serologically active and a portion corresponding to 530 C₄ was not encountered. Moreover, the acetic acid-precipitable, serologically active fractions of Lot 531 were much lower in phosphorus than the corresponding fractions of Lot 530. Since the inactive fractions associated with the serologically active, acetic acid-precipitable fractions of this lot were found to be high in phosphorus, the presence of this element in an active fraction might be taken as indicative of contamination with inactive material. The behavior of the polysaccharides of the human type cell also supports this conclusion, for Fraction 520 B₂C, which was high in phosphorus, was found to be serologically

inactive, while active fractions such as 520 C or 520 B₂ were low in phosphorus.

The possibility that most of the fractions consisted of mixtures of active and inactive material discouraged closer chemical study. Tests were made, however, for pentose and inosite. The latter was reported by Anderson and his coworkers (7-10) in carbohydrate material isolated from lipids of tubercle bacilli. Inosite, however, occurred in very few of our fractions and therefore appeared unrelated to their serological activity. On the other hand, all serologically active fractions tested were found to contain pentose, seventeen of nineteen active fractions exhibiting a strong to very strong pentose reaction (++ to +++ in Tables I to III). The inactive fractions were usually either free from pentose or contained very little, twenty-six of thirty-six fractions giving no test or only a weak pentose reaction (-, ±, or + in Tables I to III). (For the occurrence and significance of pentose in tuberculin polysaccharides from human and bovine strains cf. (11).)

Since many of the serologically inactive fractions precipitated antiserum at the higher concentrations, some of the stronger pentose tests in these fractions were undoubtedly due to the presence of active material. It seems reasonable to conclude, therefore, that the principal serologically active bovine fractions contain pentose as an essential component, in line with the actual isolation of *d*-arabinose from the corresponding fractions of the human strain (3).

SUMMARY

1. The polysaccharides extracted from the cells of a bovine strain, M-1698, of tubercle bacillus proved to be a more complex mixture of serologically active and inactive carbohydrates than was obtained from the human strain, H-37. Most of the material was serologically inactive.

2. The specific polysaccharides showed the same specificities as did those of the human type. Two independently specific polysaccharides were isolated, as well as carbohydrate combining both specificities.

3. Serologically inactive constituents consisted of at least two different strongly dextrorotatory, phosphorus-containing car-

bohydrates and one phosphorus-free substance with little optical activity. The latter and one of the phosphorylated polysaccharides were not found in the human Strain H-37.

4. Evidence is given that the serological activity of the bovine polysaccharides is connected with the presence of pentose, as in the human type strain.

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WALDEN INVERSION

XXI. THE HALOGENATION OF AROMATIC CARBINOLS. ROTATORY DISPERSION OF AROMATIC CARBINOLS AND CORRESPONDING BROMIDES*

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In recent years many efforts have been made aiming to define the conditions of the reaction of substitution which lead to inversion of configuration (Walden inversion). The investigations in this direction of Polanyi *et al.*,¹ of Olson,² of Hughes, Ingold, *et al.*³ are most outstanding.

Polanyi *et al.* and Olson came to the conclusion that all reactions of substitution by a negative group or ion are connected with inversion of configuration. On the other hand, Hughes, Ingold, *et al.* envisaged three possibilities for substitution by a negative ion: inversion, racemization, and retention of configuration, depending on the mechanism of the reaction. Reactions which follow kinetically a bimolecular course are always connected with inversion; those following a monomolecular course may be connected with inversion, racemization, or retention, the inversion always being associated with extensive racemization. These conclusions were accepted also by Steigman and Hammett.⁴ The concluding lines of the paragraph in which Hughes, Ingold,

* The two papers (Levene, P. A., Rothen, A., and Kuna, M., *J. Biol. Chem.*, **120**, 777 (1937); **121**, 747 (1937)) are Papers XIX and XX of the series on Walden inversion.

¹ Meer, N., and Polanyi, M., *Z. physik. Chem., Abt. B*, **19**, 164 (1932). Bergmann, E., Polanyi, M., and Szabo, A. L., *Tr. Faraday Soc.*, **32**, 843 (1936).

² Olson, A. R., *J. Chem. Phys.*, **1**, 418 (1933).

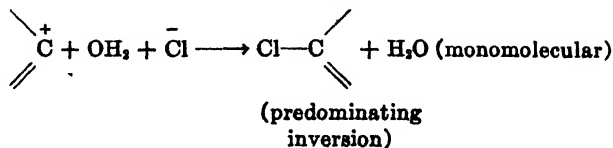
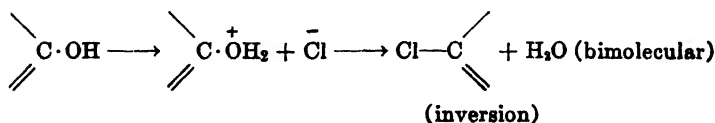
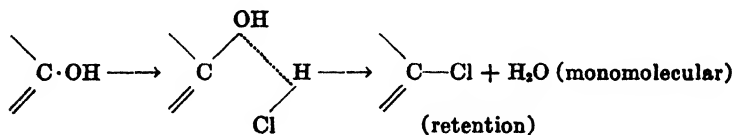
³ Cowdrey, W. A., Hughes, E. D., Ingold, C. K., Masterman, S., and Scott, A. D., *J. Chem. Soc.*, 1252 (1937).

⁴ Steigman, J., and Hammett, L. P., *J. Am. Chem. Soc.*, **59**, 2536 (1937).

et al. discussed the three possibilities, read as follows: "Since negative mechanism substitutions are always nucleophilic and bimolecular, there is agreement as to these with Meer and Polanyi's theory; and, of course, with Olson's." Indeed, so strong is the belief of the English group of investigators in their conclusions that often when kinetic data were missing the mechanism of reaction was formulated not from kinetic data but from the direction of rotation of the product of the reaction.

The halogenation of secondary carbinols having a phenyl group attached to the asymmetric carbon atom presents a peculiarity of behavior, since Levene and Mikeska⁵ found that the two lower homologues of the series (methyl- and ethylphenylcarbinols) formed, on halogenation with hydrogen bromide, a bromide rotating in opposite direction from the carbinol, whereas the higher homologues under identical conditions formed bromides rotating in the same direction.

For the halogenation of these carbinols, Hughes, Ingold, *et al.* postulated three mechanisms.

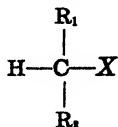


Two mechanisms result in inversion and one in retention of configuration. Satisfactory data on the kinetics of these reactions do not exist.

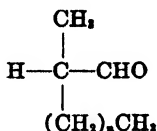
In an attempt to obtain data for the study of the kinetics of

⁵ Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **70**, 355 (1926).

such substitutions, three levorotatory carbinols, methyl-, ethyl-, and propylphenylcarbinols, were halogenated with hydrogen bromide at different temperatures. These three carbinols are configurationally related on the following grounds. Previous experience had shown that in a series of compounds of the type



homologous with respect to R_1 or R_2 , the sign of the partial rotation of a definite absorption band of the group X remains the same throughout the series; for example, the partial rotation of the aldehydic absorption band⁶ at λ 2950 in a series of aldehydes of the type



has the same sign throughout the series. The rotatory dispersion curves of the three carbinols are of the same type and there can be no doubt that these three compounds are configurationally related when of the same sign of rotation, this being true also for the three corresponding bromides.

The results of the observations on bromination are summarized in Fig. 1, where the molecular rotations of the resulting bromides are plotted against the temperature of reaction. The magnitudes of the rotations have been calculated on the basis of the reported maximum rotations of the carbinols (see the experimental part).

It can be seen that the three curves follow a parallel course, the principal difference being that the curve of the highest homologue does not cross the axis of zero rotation. For all three curves there is observed a shift of rotation towards the right as the temperature of reaction is lowered from room temperature to about -15° . The curves of the first two members show a maximum dextrorotation at -28° and the third curve a minimum levorotation in the vicinity of -20° . For temperatures lower

⁶ Levene, P. A., and Rothen, A., *J. Chem. Phys.*, **4**, 48 (1936).

than -36° the rotations of all three bromides are negative, the reaction of bromination then proceeding predominantly without inversion.⁷ It is noteworthy that between -30° and -60° the variation in the rotations of the three halides obtained as a func-

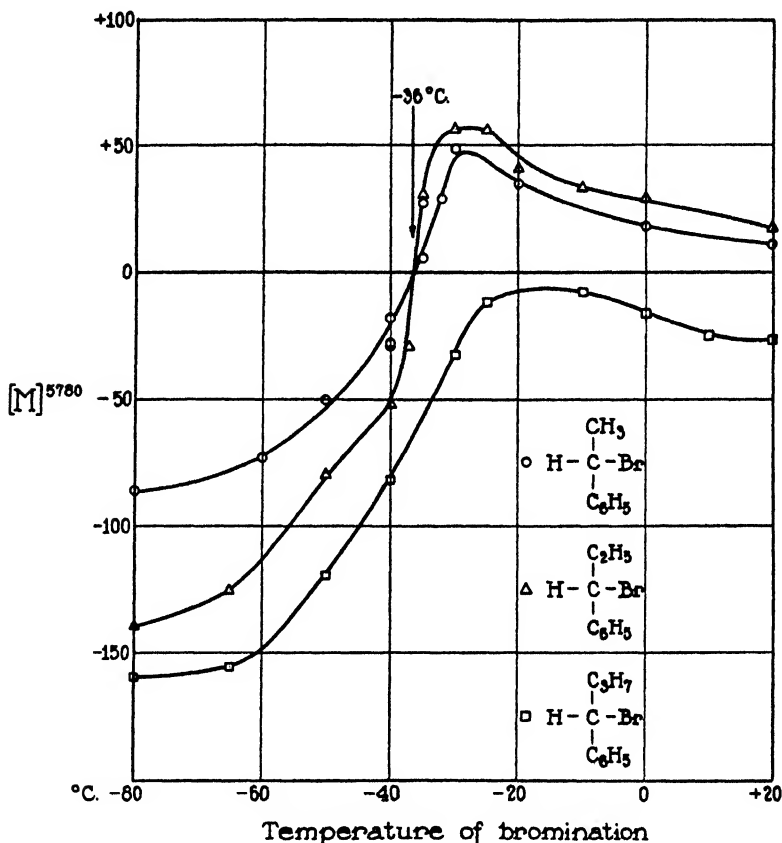


FIG. 1. Influence of temperature of bromination on the molecular rotation of the bromides obtained.

tion of the temperature of bromination is very large. It is especially striking for the second homologue, since a highly dextro-

⁷ Levene, P. A., Rothen, A., and Kuna, M., *J. Biol. Chem.*, **120**, 777 (1937).

or a highly levorotatory bromide can be obtained by varying the temperature of bromination by only a few degrees.

It is also noteworthy that the temperature of inversion (temperature of bromination at which an inactive bromide is obtained) is the same for both methyl- and ethylphenylcarbinols; *i.e.*, -36° .

It can be seen from Fig. 1 that between -60° and -80° there is observed only a small increase in the rotations of all three halides.

It is probable that the maximum values of the three bromides are only slightly greater than the values found at -80° . The following maximum values can be estimated.

Methylphenylbromomethane, $[M]_{\text{benzene}}^{5780}$	$= 90^{\circ}$
Ethylphenylbromomethane, $[M]_{\text{benzene}}^{5780}$	$= 140^{\circ}$
Propylphenylbromomethane, $[M]_{\text{benzene}}^{5780}$	$= 160^{\circ}$

The apparently opposite behavior in bromination of propylphenylcarbinol from the other two lower homologues when the bromination is carried out at room temperature is then self-explanatory in view of these curves.

The low values of the dextrorotations compared to the levorotations are due not to racemization in the ordinary sense, but actually to the independent formation of the two enantiomorphous substances. (Incidentally, the bromides, once formed, do not suffer racemization at temperatures below 0° .) Racemization occurs only at higher temperatures. The increase in levorotation of propylphenyl bromide connected with the increase of the temperature of bromination from -20° to room temperature argues against racemization being responsible for the character of the curves at temperatures below $+20^{\circ}$.

At temperatures around -30° , substitution without inversion reaches a minimum; for both higher and lower temperatures, substitution without inversion is on the increase. Hence there undoubtedly exist two different mechanisms for the reaction without inversion. One of these begins abruptly at -30° and continues towards a lower temperature. The reaction with inversion undoubtedly proceeds by a third mechanism.

Definite evidence as regards mechanism exists only for the reaction at the lowest temperature, which proceeds through an addition

product. When HBr is added to ethylphenylcarbinol at -80° , for example, a white crystalline mass is obtained which melts and decomposes sharply at -20° , a liquid bromide being formed instantly with separation of water. In the case of propylphenylcarbinol, the addition product decomposes at about -15° . If this crystalline compound is warmed rapidly to room temperature, its decomposition into halide and water takes place violently. When HBr is added to ethylphenylcarbinol at -30° it can be observed that, in addition to the solid compound, a liquid bromide is also formed. The addition product decomposes when the temperature is raised above -20° . The lower the temperature, the smaller the amount of liquid halide formed directly. In other words, for temperatures lower than -30° the amount of carbinol brominated according to the addition product mechanism becomes all the more important as the temperature is lowered.

Nothing definite can be said about the molecular structure of the addition compound. It is not impossible that the hydrogen bond complex postulated by Hughes, Ingold, *et al.* corresponds to this substance.

For temperatures above -20° , formation of this addition compound is no longer observed and hence it is justified to assume that, at higher temperature, bromination proceeds by other processes. It follows that substitution with retention of configuration is accomplished by two different mechanisms.

Consequently the mechanisms postulated by Hughes, Ingold, *et al.* do not suffice to explain the observed phenomena, since, of the three mechanisms postulated by this group of investigators, only one proceeds with retention of configuration, whereas the present observations point to two mechanisms connected with retention.

The statement of the above authors that, "There can be no doubt that, for all aliphatic, as well as the simpler aromatic, secondary alcohols, inversion is the rule," should be restricted to the saturated aliphatic compounds.

In this connection it may be stated that at the very low temperatures simple aliphatic carbinols do not react even with hydrogen iodide gas. Tertiary carbinols have not yet been tested.

Furthermore, it must be stated that these effects of temperature on the reactions here described do not appear when the reac-

tion of substitution is carried out in a solvent (aliphatic carbinols, ether, and benzene having been used as solvents). It appears from Table I that, in solution, bromination of propylphenylcarbinol follows the same course as that of the lower homologues. In all cases, whatever the temperature of bromination, the reaction proceeds with Walden inversion.

TABLE I
Bromination of Carbinols in Solution by Means of HBr

Carbinol	$[\alpha]_D^{20}$ homogeneous	Temper- ature of bromina- tion	Solvent	Concen- tration	Bromide	$[\alpha]_D^{20}$ benzene
	degrees	°C.		M		degrees
Ethylphenyl- carbinol	-37.7	-50	Ether	0.150	Ethylphenyl- bromo- methane	+79.4
"	-37.7	-80	Ethyl al- cohol	0.156	"	+135
Propylphenyl- carbinol	-69.4	-10	Ether	0.167	Propylphenyl- bromo- methane	+60
"	-69.4	-80	"	0.142 ₆	"	+88

SUMMARY

Evidence has been presented to show that in the absence of a solvent at least three different mechanisms take place in the formation of the halide from a secondary carbinol having a phenyl group attached to the asymmetric carbon atom (regardless of the size of the aliphatic radicle attached to the same carbon atom). The relative importance of each of the three mechanisms is a function of temperature. At low temperatures, for all three phenylcarbinols examined, the reaction proceeds with retention of configuration entirely through the intermediate step of an addition product.

EXPERIMENTAL

The samples of the three carbinols, methylphenyl-, ethylphenyl-, and propylphenylcarbinols, were highly resolved, thus assuring a high degree of purity. They were carefully fractionated and the constancy of the index of refraction of the different fractions was

observed. Methylphenyl- and ethylphenylcarbinols crystallized readily in solid carbon dioxide-alcohol mixture and propylphenylcarbinol crystallized immediately at room temperature.

Hydrobromic acid gas was prepared in an all-glass apparatus by allowing 100 per cent phosphoric acid to react with dry KBr.⁸ The gas was dried at -30° , passed through a tube filled with copper turnings, and condensed at -80° . It was then twice distilled and stored under pressure at 2 atmospheres in a 5 liter flask.

For each bromination experiment, a sample of about 0.1 gm. of carbinol was placed in a flask of roughly 50 cc. capacity which was sealed directly onto a glass line connected with the hydrobromic acid reservoir. The flask was then evacuated (oil pump and mercury diffusion pump) and cooled to the appropriate temperature in a mixture of solid carbon dioxide-alcohol contained in a Dewar flask equipped with a stirring device. The small amount of carbinol used as compared with the surface of the vessel was advantageous to maintain a constant temperature. An excess of hydrobromic acid gas was introduced very slowly, the rate being measured with a mercury manometer. At the end of the reaction, the flask was evacuated to remove the excess of hydrobromic acid and then brought to room temperature. The exact amount of HBr introduced was measured by the increase in weight.

It was found that a larger amount of hydrobromic acid was absorbed than that required by the stoichiometric equation, allowance being made for the very slight amount dissolved in the products of reaction. At room temperature, 1.12 moles of HBr were consumed for 1 mole of carbinol and when the reaction took place at -80° , the equivalence was 1.04 moles of HBr for 1 mole of carbinol.

The reaction was, of course, heterogeneous, HBr gas reacting with the carbinol either in the solid or liquid phase.

No study was made of the rate of the reaction, sufficient time being allowed to insure complete reaction. At room temperature the reaction was completed in a minute or so; at -80° half an hour was sufficient to produce complete bromination. For propylphenylcarbinol at least, the total reaction proceeded faster at -80° than at -40° , only 80 per cent of carbinol being brominated at -40° in half an hour.

⁸ Murray, W. J., *J. chim. physiq.*, **15**, 344 (1917).

As soon as the reaction was completed, the contents of the reaction flask were dissolved in benzene, dried rapidly with drierite, and filtered. The volume was made up to 5 cc. and the rotation measured in a 2 dm. tube. The benzene solution was then washed with a cold solution of KHCO_3 and dried with drierite. The benzene was removed by distillation and the halide distilled in a micro flask in a high vacuum (oil pump and mercury diffusion pump), the temperature of the heating bath being 35° for the lowest and 45° for the highest homologue. About 0.08 gm. of halide was generally isolated when the sample of carbinol was

TABLE II
Some Bromination Experiments

Amount	Temperature of bromination	Before distillation		Isolated bromide					
		α_{5780}	α_{5461}	Amount	α_{5780}	α_{5461}	C found	H found	n_D^{25}
Levo-ethylphenylcarbinol									
gm.	$^{\circ}\text{C.}$	degrees	degrees	gm.	degrees	degrees	per cent	per cent	
0.1180	-80	-3.644	-4.228	0.0436	-0.986	-1.138	54.35	5.67	1.5487
0.1010	-40	-1.079	-1.252	0.0395	-0.335	-0.389	54.23	5.58	1.5487
0.1042	-35			0.0124	+0.062	+0.072	54.31	5.61	
0.1049	-25	+1.371	+1.585	0.0527	+0.487	+0.564	54.28	5.67	1.5488
0.1055	0	+0.685	+0.787	0.0540	+0.256	+0.299			1.5487
Levo-propylphenylcarbinol									
0.0999	-80	-4.140	-4.790	0.0408	-1.216	-1.413	57.01	6.30	1.5408
0.1275	-25			0.0747	-0.167	-0.193	56.82	6.20	1.5408
0.0994	-10			0.0406	-0.072	-0.084	56.44	6.14	1.5410
0.1250	0	-0.559	-0.639	0.0802	-0.246	-0.282			1.5408

about 0.1 gm. This amount was sufficient for the determination of rotation, index of refraction, and for combustion. Whatever the temperature of bromination, the halides obtained were homogeneous, the index of refraction being constant to ± 0.0002 . The rotations were taken for two wave-lengths, λ 5780 and λ 5460. The theoretical value of the ratio of the rotations $\alpha_{5461}/\alpha_{5780}$ (1.160 for propylphenylbromomethane and 1.157 for ethylphenylbromomethane) was observed in each case, within experimental error.

A few examples of bromination experiments have been summarized in Table II.

In the earlier stage of the work, the benzene solution was dried overnight at 12° before isolating the halide but it was found that some racemization then occurred. If the halide were isolated a few hours after the reaction had been completed, the molecular rotation of the isolated halide was identical with that determined on the total product of reaction. (In fact, the rotation was slightly higher, 5 to 10 per cent, this being in agreement with the slight excess of hydrobromic acid absorbed, if one assumes that the products of the unimportant side reaction were not optically active.)

As already mentioned, the values of $[M]_{5780}^{25}$ given in Fig. 1 have been based on the values assumed for the maximum rotations of the carbinols which are as follows:

Methylphenylcarbinol,⁹ $[\alpha]_{5893}^{20} = 43.4^\circ$

Ethylphenylcarbinol,¹⁰ $[\alpha]_{5893}^{20} = 27.7^\circ$

Propylphenylcarbinol,¹¹ $[\alpha]_{5893}^{20} = 43.6^\circ$

We redetermined the rotatory dispersion of methylphenylcarbinol and propylphenylcarbinol. The dispersion of the latter compound was determined on two different samples prepared by two different syntheses. The data were consistent within experimental error and appreciably different from the values given by Kenyon and Partridge.¹¹ Special care was taken to have maximum precision in these determinations. Sodium arc, helium arc, and mercury arc were used as sources of light. Photographic observations were made for λ 4358 and λ 4046. Accuracy is believed to be $\pm 0.002^\circ$ for the yellow and green light and $\pm 0.01^\circ$ for the violet region. Those samples whose dispersions are reported in Table III were used for the bromination experiments.

From the figures given in Table III and from the $[M]_{5893}^{25}$ values assumed, the following simple equations were obtained to express the maximum molecular rotations in the visible spectrum, maximum deviations of 0.3 per cent occurring only in the region λ 4000.

⁹ Houssa, A. J. H., and Kenyon, J., *J. Chem. Soc.*, 2260 (1930).

¹⁰ Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 105, 1115 (1914). We wish to correct an error in this article giving $[\alpha]_{5893}^{20} = 25.86^\circ$, a value also recorded by Lowry (Lowry, T. M., *Bureau of Standards, Misc. Pub.* 118 (1932)).

¹¹ Kenyon, J., and Partridge, S. M., *J. Chem. Soc.*, 128 (1936).

$$\text{Methylphenylcarbinol, } [M]_{\text{homogeneous}}^{\text{D}} = \frac{13.24}{\lambda^2 - 0.0425}$$

$$\text{Propylphenylcarbinol, } [M]_{\text{heptane}}^{\text{D}} = \frac{14.92}{\lambda^2 - 0.032}$$

The maximum values of propylphenylcarbinol in benzene solution are 14 per cent greater than in heptane and the dispersion constant has the value 0.0345.

TABLE III

Rotatory Dispersion of Phenylcarbinols in Homogeneous State, $l = 10$ Cm.

Compounds	n_D^{25}	α_{589}^{25}	α_{578}^{25}	α_{570}^{25}	α_{541}^{25}	α_{485}^{25}	α_{435}^{25}
		degrees	degrees	degrees	degrees	degrees	degrees
Methylphenylcarbinol.....	1.5253	-32.470		-33.966	-38.753	-67.21	-81.49
Ethylphenylcarbinol.....	1.5180			-23.413	-26.560		
Propylphenylcarbinol in heptane*.....			-13.719	-14.240	-16.170	-27.235	-32.53

* Concentration, 0.7259 M, $l = 40$ cm.

TABLE IV

Rotatory Dispersions in Homogeneous State of Phenylalkylhalogenomethanes

Compounds	d_D^{25}	n_D^{25}	α_{578}^{25}	α_{570}^{25}	α_{541}^{25}	α_{485}^{25}	α_{435}^{25}
			degrees	degrees	degrees	degrees	degrees
Ethylphenylbromomethane, $l = 10$ cm...		1.5493	50.953	53.121	61.602	117.71	149.96
Propylphenylmonomethane, $l = 10$ cm...	1.263 ₁	1.5413	15.358	16.006	18.542	35.28	44.94
Ethylphenylchloromethane, $l = 20$ cm...	1.034 ₂	1.5196	40.348	42.018	48.370	88.55 ₁	110.56
Propylphenylchloromethane, $l = 10$ cm...	0.990 ₄	1.5132	5.319	5.525	6.349	11.55	14.30

The rotatory dispersion of two of the bromides has been determined and, as already mentioned, the practical identity of the dispersion constants of a one Drude term formula, $\lambda_0^2 = 0.071$ for ethylphenylbromomethane and $\lambda_0^2 = 0.070$ for propylphenyl-

bromomethane, indicates that the compounds are configurationally related when they have the same sign of rotation.

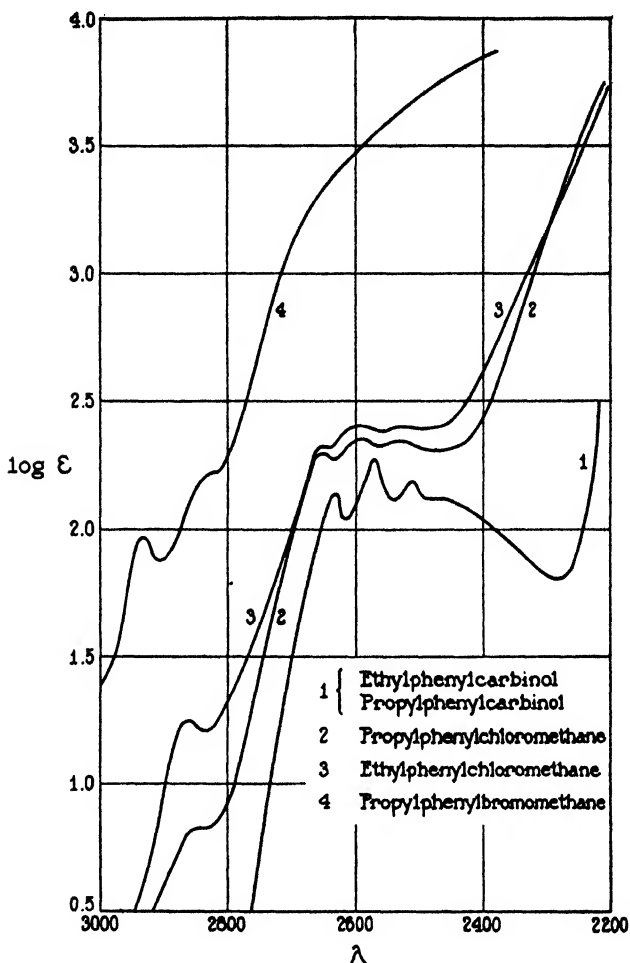


FIG. 2. Absorption spectra of halides. The log of the molecular extinction coefficient is plotted against the wave-length.

Similarly, the dispersion constants of the corresponding chloro derivatives are very close, the lower homologue, as in the case of the bromides, having a slightly greater value ($\lambda_0^2 = 0.0597$ for

ethylphenylchloromethane and $\lambda_0^2 = 0.0578$ for propylphenylchloromethane).

The dispersion data have been summarized in Table IV.

Absorption curves can be seen in Fig. 2. The solvent used for all compounds was heptane.

The curves for the two chloro derivatives are not so close as might have been expected, but it should be remembered that these substances are very unstable towards ultraviolet light; therefore we do not consider these discrepancies to be significant.

Some resolution of the absorption region λ 2620 to λ 2500 of the phenyl group is apparent in the carbinols but has practically disappeared in the chloro and is unobservable in the bromo derivatives.

This absorption region is not anisotropic in the case of the carbinols and the chloro derivatives, as is apparent from the rotatory dispersion data.

On the contrary, it would seem from the value of the dispersion constant of the bromo derivatives ($\lambda_0^2 \simeq 0.070$) that this absorption region is anisotropic in that case, but it should not be forgotten that the dispersion curve is of the type represented by two Drude terms of opposite sign (as is already apparent from the dispersion data in the visible region, the calculated value of α_{4046} being larger than the observed value of α_{4046}). The position of the first active absorption band is probably much lower in the wave-length scale than λ 2600.

ENZYMATIC DEPHOSPHORYLATION OF DESOXYRIBO- NUCLEIC ACIDS OF VARIOUS DEGREES OF POLYMERIZATION*

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PLATE 4

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It has been observed^{1,2} that the rate of enzymatic hydrolysis of thymonucleic acid differs very considerably for different samples. Inasmuch as it was known that small changes in the methods of preparation might affect the degree of polymerization of the resulting product, it seemed desirable to investigate the effect of enzymes on nucleic acids which were known to differ in the degree of their polymerization.

Four types of thymonucleic acid were selected for the experiments. The types differed in the methods of their preparation; namely, those prepared according to Hammarsten,³ Neumann⁴ (modified by Feulgen⁵), Levene,⁶ and Feulgen⁷ (enzymatic), respectively.

To date, molecular weight data of a fair degree of accuracy exist only in the case of the acid of the first type, leading to a molecular weight of approximately 1 million.⁸ To this information are now

* The term "phosphatase" is used in this paper for the sake of brevity. It is self-evident that the material contains a complex of enzymes.

¹ Levene, P. A., and Dillon, R. T., *J. Biol. Chem.*, **88**, 753 (1930).

² Schmidt, G., *Enzymologia*, **1**, 135 (1936).

³ Hammarsten, E., *Biochem. Z.*, **144**, 383 (1924).

⁴ Neumann, A., *Arch. Anat. u. Physiol., Physiol. Abt.*, 374 (1898); suppl., 552 (1899).

⁵ Feulgen, K., *Z. physiol. Chem.*, **90**, 261 (1914).

⁶ Levene, P. A., *J. Biol. Chem.*, **53**, 441 (1922).

⁷ Feulgen, K., *Z. physiol. Chem.*, **233**, 105 (1936).

⁸ Signer, R., Caspersson, T., and Hammarsten, E., *Nature*, **141**, 122 (1938). Astbury, W. T., and Bell, F., *Nature*, **141**, 747 (1938).

added data concerning the sodium salts of the second and fourth types. Of these two, the former sedimented with a blurred boundary, indicating particles of molecular weights ranging from about 50,000⁹ to at least 1 or 2 million, while no appreciable sedimentation could be observed with the latter. Thus the descending order of polymerization is (1) thymonucleic acid of Hammarsten, (2) that of Neumann, (3) that of Levene, and (4) that of Feulgen (by the enzymatic process).

It has now been found that the increase in the degree of enzymatic dephosphorylation follows the same order, Hammarsten's material being the most stable. When purified enzyme was employed, no dephosphorylation of this acid was observed even when the duration of the experiment was extended to 4 hours, whereas the duration of the experiment with the nucleic acids of the other types was limited to 1 hour. The highest degree of dephosphorylation was observed with the material obtained by the enzymatic method of Feulgen.

The slight degree of dephosphorylation observed on treatment of Hammarsten's nucleic acid with crude intestinal extract was undoubtedly due to the presence in it of a certain amount of "depolymerase." In harmony with this assumption is the observation that nucleic acids of low degree of polymerization are dephosphorylated to approximately the same degree by the crude or purified enzymes, whereas those of the highest degree of polymerization are dephosphorylated by the crude enzyme only. The observation that the samples of higher degree of polymerization exhibit no inhibitory action on the dephosphorylation of the acids of lower degree of polymerization is likewise in harmony with the view that dephosphorylation of Hammarsten's nucleic acid by the crude enzyme is to be attributed to the presence of a depolymerase.

In the light of the present observations, a new interpretation should be given to Schmidt's² earlier observation that the enzymatic dephosphorylation of nucleohistone is enhanced by preliminary digestion with pancreatin. The earlier explanation postulated a hydrolysis of the nucleohistone into histone and nucleic acid prior to dephosphorylation. This assumption should be revised in

⁹ As discussed later in the text, this figure represents the minimum possible molecular weight.

favor of the depolymerizing effect of pancreatin on the nucleic acid. This interpretation is also in harmony with the observation that only pancreatin (and not *purified* trypsin) enhances the enzymatic dephosphorylation of nucleohistone.

EXPERIMENTAL

Methods

Determination of Phosphoric Acid—The trichloroacetic acid filtrate of the digestion product was made alkaline by means of ammonia, precipitated with Mathison's¹⁰ magnesia mixture, and the phosphoric acid determined according to the method of Embden.¹¹

Preparation of Intestinal Enzyme—Glycerol extracts from calf duodenum and jejunum, prepared according to the method of Klein,¹² were employed as starting material for the purification of the enzyme. About 80 per cent of the glycerol was then removed by dialysis through cellophane in a cold room at 10° (1000 ml. of extract against 10 volumes of distilled water which was changed three times, the time of dialysis being 15 to 20 hours).

Following this stage of the preparation, two different procedures were found suitable for obtaining the phosphatase free from the depolymerase.

Procedure A—The dialyzed extract was concentrated under diminished pressure to one-tenth of its volume and again dialyzed overnight to remove the glycerol. When the dialyzed solution was kept for several hours at room temperature, a precipitate (which contained the larger part of the phosphatase activity) settled out. The sediment was centrifuged, washed once with water, and dissolved by suspending it in water and carefully adding a few drops of dilute ammonia. This solution kept its activity at 0° for 2 days.

Procedure B—To each 1000 ml. of the dialyzed extract were added 100 to 150 ml. of an aluminum hydroxide suspension, dropwise with vigorous stirring. The procedure was ended when a sample of the centrifugate gave only a slight opalescence with trichloroacetic acid. After removal of the aluminum hydroxide

¹⁰ Mathison, G. C., *Biochem. J.*, **4**, 233 (1909).

¹¹ Embden, G., *Z. physiol. Chem.*, **113**, 138 (1921).

¹² Klein, W., *Z. physiol. Chem.*, **207**, 125 (1932).

by centrifuging, the supernatant liquid was concentrated under diminished pressure to one-tenth of its volume and again dialyzed for a few hours in order to remove the remaining glycerol. This solution, which retained 60 to 80 per cent of the original phosphatase activity, was used for the experiments.

The aluminum hydroxide suspension was prepared by adding ammonium hydroxide solution to a 5 per cent solution of aluminum sulfate at boiling temperature. The precipitate was washed until the supernatant liquid turned slightly opalescent.

Remarks—Procedure B is similar to the method of Schöffner for the preparation of yeast phosphatase.^{13,14}

For large scale preparation of the enzyme, the use of glycerol involves the inconvenience of dialyzing large volumes of liquid. However, in the absence of glycerol, aqueous solutions of phosphatase were found to be unstable.

Test for Phosphatase Activity—5 cc. of a 2 per cent sodium thymonucleinate solution, 1 cc. of approximately N ammonia-ammonium acetate buffer (pH 9.0), and 2 cc. of the enzyme solution were mixed and diluted to a volume of 20 cc. At the end of the incubation, 5 cc. of a 20 per cent solution of trichloroacetic acid were added. An aliquot part of the filtrate was used for the phosphorus determination.

Table I contains the results of six series of experiments, each series having been repeated several times with the same results. The enzyme solutions used in individual series differed in their method of preparation but the identical enzyme solution was used for all substrates investigated in any one series.

Series 1—The enzyme used was a suspension of fresh mucosa in 2 per cent sodium bicarbonate solution (1:5).

10 cc. of 1 per cent solution of sodium nucleinate + 1 cc. of the N buffer solution + 2 cc. of magnesium acetate (1 per cent) + 5 cc. of enzyme suspension were allowed to digest 1 hour at 37°.

Series 2—The enzyme used was the precipitate obtained accord-

¹³ Schöffner, A., Bauer, E., and Berl, H., *Z. physiol. Chem.*, **232**, 213 (1935).

¹⁴ The lack of adsorption affinity towards aluminum hydroxide seems to be a property characteristic of the alkaline phosphatase, since enzyme preparations from such different sources as intestine, liver, and yeast show the same behavior in this respect.

ing to Procedure A. Digestion was as in Series 1. The amount of enzyme was equivalent to that in Series 1 (calculated for fresh mucosa).

TABLE I
Effect of Phosphatase Preparations on Various Types of Thymonucleic Acid

Series No.	Phosphatase	Substrate*	P released	
			mg.	per cent from total P in substrate
1	Crude	Thymonucleic acid (H.)	0.73	12.2
		" " (N.)	1.17	17.0
		" " (L.)	1.45	22.3
		" " (F.)	3.60	57.6
		Glycerophosphate	4.41	63.0
2	Purified	Thymonucleic acid (H.)	0	0
		" " (N.)	0.60	8.3
		" " (L.)	1.43	21.6
		" " (F.)	2.94	46.8
		Glycerophosphate	4.00	57.7
3	" in absence of Mg	Thymonucleic acid (H.)	0	0
		" " (N.)	0.75	10.9
		" " (F.)	3.65	57.3
4	Purified	" " (H.)	0	0
		" " (F.) (precipitated)	5.23	83.2
5	"	Thymonucleic acid (H.)	0	
		Same after digestion with pancreatin	4.6	
6	Crude (dialyzed glycerol extract)	100 mg. thymonucleic acid (H.)	0.22	
		100 " " " (F.)	2.30	
		100 " " " (H.)	2.22	
		+ 100 mg. thymonucleic acid (F.)		
		100 mg. thymonucleic acid (H.) + 50 mg. glycerophosphate	3.44	

* The letters H., N., L., and F. in parentheses represent Hammarsten's, Neumann's, Levene's, and Feulgen's thymonucleic acid respectively.

Series 3—The enzyme used was the precipitate obtained according to Procedure A, 10 cc. of the enzyme solution corresponding to 2.5 cc. of glycerol extract.

5 cc. of 2 per cent sodium nucleinate solution + 1 cc. of the buffer solution + 10 cc. of enzyme solution were allowed to digest 1 hour at 40°.

Series 4—Feulgen's nucleic acid was reprecipitated from a 20 per cent solution by adding a sufficient amount of 20 per cent hydrochloric acid and $\frac{1}{2}$ volume of 95 per cent alcohol. The precipitate was washed and then dissolved in water by neutralization with sodium hydroxide. Precipitation of the sodium salt was accomplished by adding 4 volumes of 95 per cent alcohol. The conditions of the experiment were as in Series 3; duration, 2 hours at 40°.

Series 5. Depolymerization of Hammarsten's Thymonucleic Acid—5 gm. of nucleinate were dissolved in 100 cc. of 1 per cent pancreatin solution (Merck). The solution was incubated 2 days at 37°. The reaction product was precipitated with 500 cc. of 95 per cent alcohol. Yield, 4.5 gm. (The precipitate was no longer fibrous.) It was reprecipitated as in Series 4.

The enzyme used was dialyzed glycerol extract, purified by treatment with $\frac{1}{2}$ volume of aluminum hydroxide suspension. The conditions of the experiment were as in Series 3; duration, 3 hours at 37°.

Series 6—The enzyme used was dialyzed glycerol extract (the amount in each sample corresponding to 0.7 cc. of crude extract). The conditions of the experiment were as in Series 2.

Sedimentation Experiments

The sedimentation of Feulgen's and of Neumann's nucleic acids in the form of their sodium salts was investigated in the analytical ultracentrifuge of Bauer and Pickels.¹⁵

0.05 per cent solutions of the sodium nucleinates were used, to which 1 per cent sodium chloride was added to minimize electrical influences. The sedimentations were recorded photographically by the light absorption method of Svedberg, ultra-violet light (2536 Å.) being used for the illumination. Each solution was subjected to a mean centrifugal force 210,000 times gravity at 54,000 R.P.M. for 35 minutes.

As shown by the photographs in Fig. 1, the molecules of Feul-

¹⁵ Bauer, J. H., and Pickels, E. G., *J. Exp. Med.*, **65**, 565 (1937).

gen's nucleic acid were apparently so small that no appreciable sedimentation occurred. As illustrated in Fig. 2, however, the particles of Neumann's nucleic acid sedimented quite readily, although at varying rates. A comparison of Figs. 1 and 2 suggests a manifold difference in the average molecular weights of the two types of nucleic acid. This conclusion is in agreement with the observations that Neumann's nucleic acid shows a considerably higher viscosity and a great tendency to gelatinize.

The very blurred appearance of the sedimenting boundary for Neumann's substance (Fig. 2) is most probably due to a wide range of particle size. The majority of the particles sedimented at rates lying between 8 and 100×10^{-13} cm. per sec. per dyne, the minimum rate being far better defined than the maximum. These values indicate, in accordance with Stokes' law, that the smallest molecules of Neumann's sodium nucleinate have a molecular weight of at least 50,000¹⁶ and that some of the particles have molecular weights considerably above 1 million. The actual weights, which may be considerably greater, could not be computed, since information regarding the shape or diffusion coefficients of the particles is not available.

Nothing definite can be stated about the molecular weight of Feulgen's nucleinate. The fully depolymerized substance has a molecular weight of 1341.

Because of the very strong absorption of ultraviolet light by the nucleinates, their sedimentation in concentrations above 0.05 per cent was not investigated. Whether an increase in the concentration would lead to an association of particles remains an open question.

For estimating these molecular weights from centrifugal data, it was necessary to determine the approximate density of sodium nucleinate particles in water. This was done by the pycnometer method for Hammarsten's, Neumann's, and Feulgen's substances. In each instance, the amount of residual water present in the stock

¹⁶ In a preliminary note (Schmidt, G., and Levene, P. A., *Science*, **88**, 172 (1938)), a rough estimate of the minimum molecular weight was given as 200,000. For this calculation the density was taken to be approximately the same as that of most proteins and sugars; namely, 1.3 to 1.4 gm. per cc. Our own recent determinations, as given in the text, indicate a higher value for the density of the nucleinates.

material, which had been prepared by desiccating at room temperature, was determined by drying a sample to constant weight at 110°. With all three preparations a density value of approximately 2 gm. per cc. was found for the dry material (Hammarsten's nucleinate 2.15 gm. per cc., Neumann's nucleinate 1.75 gm. per cc., Feulgen's nucleinate 1.81 gm. per cc.). The high values for the densities of the sodium nucleinates are in agreement with the general behavior of sodium salts as compared with that of the corresponding free organic acids. The densities of solutions of the free nucleic acids in water cannot be determined because of their insolubility.

However, we have estimated the density of a 1 per cent solution of thymonucleohistone and obtained the value of 1.41 gm. per cc. which is only slightly higher than the density of most proteins (1.33 gm. per cc.).

SUMMARY

1. Sedimentation studies revealed that sodium desoxyribonucleinate prepared by different methods shows a range of molecular weights approximately between 1500 and above 1 million. The acid prepared by Neumann's method consists of a mixture of substances of varying degrees of polymerization.

2. Native desoxyribonucleic acid is not dephosphorylated by purified intestinal extracts.

3. Desoxyribonucleic acid, depolymerized by Feulgen's method and further purified, is dephosphorylated by crude and by purified enzyme to approximately the same extent.

4. Desoxyribonucleic acid containing a considerable proportion of native nucleic acid is dephosphorylated to different degrees by crude and purified enzyme.

5. The state of polymerization of desoxyribonucleic acid can be appraised by determining its reaction to pure and crude intestinal extract.

6. It is not as yet known whether the variations in the stability of the nucleic acids as a function of degree of polymerization are to be attributed to differences in their susceptibility to the action of phosphatase or of polynucleotidase. This point will be made the topic of a separate study.

Addendum—In a recent publication, Bredereck, Caro, and Richter¹⁷ attribute to Levene and Dillon a statement to the effect of the existence in the gastrointestinal secretion of the dog of two different nucleinases, one specific for ribo- and the other for deoxyribonucleic acid.

This conception they refute by the finding that a phosphatase obtained from sweet almonds acts with the same velocity on the two nucleic acids.

In no place have Levene and Dillon made the statement attributed to them. Furthermore, the enzyme obtained from sweet almonds active at pH 4.9 to 5.1 has no bearing on the nucleases of gastrointestinal origin active at pH 8.5 to 9.

EXPLANATION OF PLATE 4

FIG. 1. Photographs taken with the ultracentrifuge showing the failure of Feulgen's nucleinate to sediment appreciably in a centrifugal field 210,000 times gravity. The pictures were taken at 5 minute intervals.

FIG. 2. Sedimentation photographs of Neumann's gelatinizing nucleinate in a centrifugal field 210,000 times gravity, showing the settling of particles with a blurred boundary. The pictures were taken at 5 minute intervals.

¹⁷ Bredereck, H., Caro, G., and Richter, F., *Ber. chem. Ges.*, **71**, 2389 (1938).

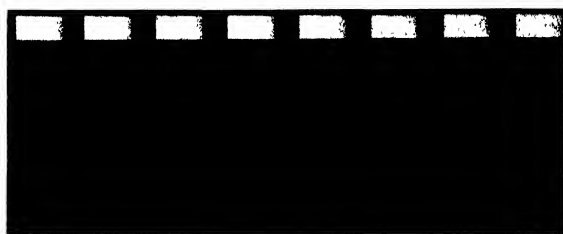


FIG. 1



FIG. 2

QUANTITATIVE INVESTIGATIONS OF AMINO ACIDS AND PEPTIDES

V. THE FUNCTION OF IODINE IN AMINO NITROGEN ANALYSES BY THE NITROUS ACID METHOD

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(Received for publication, October 7, 1938)

A modification of the Van Slyke manometric amino nitrogen method by which theoretical results are obtained with glycine and cystine is described in a recent paper by Kendrick and Hanke (1). The amino nitrogen values for these amino acids are brought to the normal level by means of a nitrous acid-acetic acid reagent to which iodide has been added.

Because iodine was found to be as effective as iodide in giving theoretical values for glycine and cystine, it was assumed that the abnormal reducing groups (sulfide sulfur of cystine (2, 3) and the deaminized glycine molecule (3)) were oxidized by iodine before they could react appreciably with nitrous acid. As support for this theory, it was reported that iodide, when mixed with nitrous acid, is immediately oxidized to iodine and that the cystine abnormality is not eliminated by iodide when the deaminizing mixture contains thiosulfate. Practically no iodine is formed under these conditions.

On the other hand, the fact that the ferric-ferrous system, which has approximately the same oxidation-reduction potential as the iodine-iodide system, had no effect on the nitrogen values from the reaction of cystine and nitrous acid stands in opposition to this theory. The further observation that sulfate is formed when cystine is treated with nitrous acid in the presence, as well as in the absence, of iodide appears to have no critical significance.

In other experiments, Kendrick and Hanke found that leucine, alanine, glutamic acid, lysine, histidine, and tyrosine yield iden-

tical values with, or without, iodide. It was stated, however, that, "Tryptophane is one amino acid yielding non-theoretical values, 40 to 60 per cent, when KI is used."

Comparative studies of blood filtrates led these authors to the conclusion that the "KI methods yield consistently lower results, by 8 to 15 per cent, than do Van Slyke's and other non-KI methods."

It is evident from the present authors' experimental data, given in Table I, that the results reported by Kendrick and Hanke for leucine, cystine, and tryptophane, *but not for glycine*, are confirmed. It was of interest, therefore, to give further consideration to the probable explanation for the iodide effect.

It is difficult to accept the theory that iodine preferentially oxidizes cystine sulfide and deaminized glycine. Iodine is a weaker oxidizing agent than nitrous acid at comparable concentrations (see data given in Table II) and there should be greater divergence in the oxidizing capacities of these agents in the experimental solutions which contained iodine in less than 0.01 the molal concentration of the nitrous acid. In order to test the possibility that the rate of oxidation by iodine might be faster than that by nitrous acid, separate solutions were prepared containing cystine and iodine in 100 times, and nitrous acid in 2 times, the molal concentrations of these substances in the experimental solutions. It was found that sulfate (tested as barium sulfate) was formed in easily detectable amounts when a mixture of equal volumes of the cystine and nitrous acid solutions had stood for 30 seconds but that no sulfate was formed in 60 minutes when the iodine and cystine solutions were tested similarly.

A clue to what is thought to be the correct explanation for the iodide effect was found during the analysis of methionine. The amino nitrogen values obtained by the use of freshly prepared iodide solution were approximately theoretical, but they fell progressively to the minimum figure of approximately 94 per cent of the theoretical amount as the iodide solution aged during a period of 1 week. It was noted, too, that the quantity of iodine in the stock iodide solution increased during this period, presumably owing to atmospheric oxidation. In order to verify the assumption that iodine is the factor indirectly responsible for the iodide effect, an analysis of methionine was made with a freshly

TABLE I
Effect of Iodide upon Amino Nitrogen Values

Amino acid*	Amino N, per cent of theoretical amount		
	Manometric method		Values by volumetric method without KI reported in literature
	Without KI	With KI	
<i>d</i> -Arginine monohydrochloride	100.0 (4 min.) 111.5 (20 ")	102.1 (6 min.)	104-150† (3)
<i>l</i> -Cystine	128.7	99.6 (5 ") 101.0 (10 ") 99.4 (15 ")	109 (4) 140‡ (1) 111-131† (3)
Cysteine hydrochloride	128	100	113† (3)
Glycine	102.9§	103.1§	103 (4) 105.5-108 (5) 108-117† (3)
<i>l</i> -Leucine¶	100.0	99.8	100 (6)
<i>dl</i> -Methionine	100.2	99.2	100 (7)
<i>l</i> -Tryptophane	99.6	48.9	118-194† (3)
<i>dl</i> -Serine	103.4	99.8	105-108** (3)
Glutathione	194-253 (4-10 min.)	204 (4 min.)	Higher than theoretical (8)

* The glutathione, cysteine hydrochloride, and *l*-tryptophane were Pfanstiehl Chemical Company's products. The purity of the glutathione and the cysteine hydrochloride was found to be 97.1 and 93.4 per cent, respectively, according to iodometric analysis by the method of Virtue and Lewis (9). According to Shinohara (10), *l*-cystine is the principal impurity in cysteine hydrochloride and it may be present in commercial samples by as much as 10 per cent by weight. The assumption that cystine and cysteine respond similarly to the iodide effect serves to explain the analytical data reported for the latter compound. The other amino acids were prepared by Amino Acid Manufactures. Their purity was 99.8 ± 0.3 per cent according to Volhard analysis and analyses by the formol titration-glass electrode method of Dunn and Loshakoff (11). The figures quoted are averages of two, three, or four runs which showed maximum deviations of 0.8 per cent.

† 4 to 6 minutes at 45°.

‡ Manometric method.

§ Average of four determinations.

|| 1 to 5 minutes.

¶ Freed from methionine by the method of Fox (12).

** 4 to 30 minutes at 45°.

prepared solution of potassium iodide saturated with iodine. The expected figure for amino nitrogen, approximately 94 per cent of the theoretical amount, was obtained.

The further observation was made that a red precipitate, thought to be mercuric iodide, appeared in the manometric deaminizing apparatus when 1 week-old potassium iodide solution was used for the analysis. As may be inferred from the data in Table II, nitrous acid is capable of oxidizing mercury to the mercurous and mercuric forms. In view of these circumstances it seems reasonable to postulate that the iodide effect with methionine, cystine, cysteine, and tryptophane may be due to the formation of slightly soluble, or feebly ionized, complexes of mercuric iodide with the sulfur, nitrogen, or other atoms of these molecules. In the case of soluble complexes, the reaction of nitrous acid with

TABLE II
Molal Reduction Potentials

Couple	E_0 , volt
$3\text{I}^- \rightleftharpoons \text{I}_3^- + 2e$	-0.54
$\text{NO} + \text{H}_2\text{O} \rightleftharpoons \text{HNO}_2 + \text{H}^+ + 2e$	-0.98
$2\text{Hg} \rightleftharpoons \text{Hg}_2^{++} + 2e$	-0.80
$\text{Hg}_2^{++} \rightleftharpoons 2\text{Hg} + 2e$	-0.92
$\text{Fe}^{++} \rightleftharpoons \text{Fe}^{+++} + e$	-0.74

the primary amine nitrogen would proceed normally but the abnormal production of nitrogen by the reaction of nitrous acid with other groups would be prevented. When the values for amino nitrogen are reduced below the theoretical amount, it may be assumed that insoluble complexes are formed.

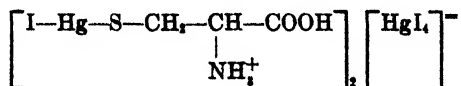
If the iodide effect is due to the formation of mercuric iodide complexes, analyses of the "abnormal" amino acids in the Van Slyke volumetric apparatus should yield identical amino nitrogen values with, and without, iodide in the deaminizing reagent. Tests made on cystine with iodide-free, and iodide-containing, reagents gave identical amino nitrogen values (112.0 and 111.8 per cent of the theoretical amount). These figures agree closely with those reported for cystine from 4 minute runs with the volumetric apparatus (3). On the other hand, approximately the theoretical value was obtained when cystine was allowed to re-

act with the nitrous acid-acetic acid reagent to which a solution of the complex salt, K_2HgI_4 (mercuric iodide in freshly prepared potassium iodide solution), had been added.

The fact that complexes are readily formed from mercuric halides and the amino acids under consideration is well known. Examples of these and related types of complexes are those which have been shown to be formed from alkyl sulfides and mercuric iodide (13) but not from alkyl sulfides and mercurous salts (14), from dithiodihydracrylic acid and mercuric bromide (10, 15), from cystine (as cysteine) and mercuric chloride (10, 15), from tryptophane and mercuric sulfate (16), and from methionine and mercuric acetate (17) or methionine and mercuric chloride (18). The apparent failure of glycine to form complexes with mercury salts is in harmony with the authors' experimental results and explanation for the iodide effect.

The exceptionally low values for amino nitrogen obtained in the analyses of tryptophane may be explained by the formation of an insoluble addition product with iodine. This would appear to be a reasonable hypothesis, inasmuch as Neuber and Nikolaus (19) prepared and analyzed comparable insoluble chlorine and bromine addition products.

The authors' attempts to isolate the complex assumed to be formed from cystine and mercuric iodide were unsuccessful. A solution of an equimolar mixture of cystine and potassium mercuric iodide was evaporated in a current of air. The yellow crystalline product was dissolved in alcohol, the solution filtered to remove undissolved cystine, and the filtrate evaporated to dryness in a current of air. The hygroscopic residue was taken up in acetone and, when the solution was cooled, a white crystalline substance precipitated. This product could not be dried without decomposition. It may be assumed from the observations of Preisler and Preisler (15) and those of Andrews and Wyman (20) on the mercuric chloride and mercuric sulfate complexes of cystine, however, that the corresponding mercuric iodide complex would have the structure



The isolation of the complexes of methionine and tryptophane was not attempted.

Kendrick and Hanke state that, "Since the iodide, when mixed with nitrous acid, is immediately oxidized to iodine, one may conclude that it is the oxidizing action of the iodine, rather than the reducing action of the iodide, which is the effective agent. This is supported by the observation that if in place of 1 cc. of 20 per cent (1.2 N) KI, 1 cc. of 0.6 N KI and 0.6 N $\text{Na}_2\text{S}_2\text{O}_3$ is used, under which conditions practically no iodine is liberated by the nitrous acid, the values with cystine are about 140 per cent, the same as by Van Slyke's method in which no iodide is used." The present authors' deductions are somewhat different from those advanced by Kendrick and Hanke.

It may be assumed that iodide is oxidized immediately to iodine by nitrous acid but, under the experimental conditions, this reaction would not proceed to completion. The evidence reported in the present paper would seem to substantiate this conclusion. It appears probable, also, that "practically no iodine is liberated by nitrous acid" from iodide when thiosulfate is present, although this observation has no critical significance if the present authors' explanation of the iodide effect is correct.

That the amino nitrogen values are about 140 per cent of the theoretical amount when cystine is analyzed by means of a deaminizing reagent containing both iodide and thiosulfate is thought to be the net result of two competing reactions. The formation of the cystine-mercuric iodide complex should proceed as well in the presence, as in the absence, of thiosulfate. The amino nitrogen values for cystine should tend to approach the theoretical because of this factor. On the other hand, thiosulfate is capable of reducing nitrous acid to nitrogen, as was demonstrated in an experiment performed by the present authors. It was found that approximately 50 ml. of nitrogen were formed from the reaction of nitrous acid with 1 ml. of 0.6 N thiosulfate in the deaminizing chamber of the Van Slyke manometric apparatus. The amino nitrogen values found for cystine would be higher than the theoretical amount, therefore, when thiosulfate is present.

The observations of Kendrick and Hanke that the amino nitrogen of blood filtrates, measured by means of the potassium iodide-manometric method, is lower by as much as 15 per cent than the

values obtained by non-potassium iodide methods was confirmed by the authors' experiments with beef blood filtrates. Similar analyses carried out with the volumetric apparatus and potassium mercuric iodide-containing reagents gave results which did not differ significantly from those obtained with reagents lacking this complex. A satisfactory explanation of these observations cannot be given at the present time.

Whether or not amino nitrogen values of blood filtrates obtained by the use of iodide-containing reagents are to be preferred to those found with non-iodide reagents is an important point. According to present knowledge, the amino nitrogen in human blood filtrates, measured by the manometric iodide method, is approximately 15 per cent less than the average 0.010 gm. per 100 ml. (21) found in analyses by manometric non-iodide methods. It may be assumed from the observations of Kendrick and Hanke, and those of the present authors, that only cystine and tryptophane of the amino acids in blood can be mainly responsible for this effect. It seems probable, however, that cystine is a relatively unimportant factor in this connection because its concentration in blood is negligible (22, 23). On the other hand, it can be readily shown by a simple calculation that tryptophane, which is present in blood in a concentration of approximately 1.0 mg. per 100 ml. (23), is the source of only about 2.5 per cent of the 15 per cent decrease in blood amino nitrogen.

It may be deduced, therefore, that reducing substances, other than amino acids, are the principal cause of the observed decrease in blood amino nitrogen. Substances of interest are glucose, ascorbic acid, glutathione, and thioneine (ergothioneine). A precise evaluation of the effect of these substances upon the amino nitrogen in blood filtrates, measured by iodide and non-iodide methods, cannot be made at the present time, because their concentration varies widely with different protein-precipitating agents.

It is known, however, that the concentration of glucose, ascorbic acid (24), glutathione (25), and thioneine (26) in blood is approximately 80, 1, 7.5, and 5 mg. per 100 ml., respectively. Although no data are available for ascorbic acid and thioneine, the authors' experiments show that about 1 and 10 per cent, respectively, of the total amino nitrogen measured by non-iodide methods could be accounted for by reactions of glucose and glutathione

with nitrous acid. It has been shown further that amino nitrogen from glutathione is unaffected by the presence of iodide in the deaminizing reagent.

Experiments which the authors plan to undertake may throw additional light on these problems.

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A RAPID MICROMETHOD FOR THE QUANTITATIVE ESTIMATION OF SUGAR ALCOHOLS

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In studying the metabolism of sorbitol and mannitol in experimental animals (1) it is desirable to have at hand a rapid method for the estimation of these compounds. Of the various methods found in the literature none was deemed satisfactory for rapid routine analysis. A method was therefore developed which lends itself to rapid and fairly accurate estimations of sugar alcohols. The method has been applied to the determination of sorbitol, mannitol, dulcitol, erythritol, pentaerythritol, and inositol, and should be applicable to the other sugar alcohols. The reactions involved are not specific for these compounds and consequently the method, though satisfactory for the work to which we have adapted it, has distinct limitations.

The principles of the Hagedorn-Jensen sugar method have been used with modifications in technique and reagents to meet special requirements. The method described below represents the best procedure so far developed.

Method

Reagents—

1. 1.08 per cent potassium ferricyanide in water.
2. 5 per cent sodium sulfate in 3.33 N sodium hydroxide.
3. 15 per cent zinc acetate and 12 per cent potassium iodide in combined solution.
4. Glacial acetic acid.
5. 0.005 N sodium thiosulfate.

Procedure

Pyrex tubes (25 × 200 mm.) are charged with 5 ml. of a solution containing 0.1 to 0.7 mg. of sugar alcohol. Water blanks are run concurrently. 3 ml. of Reagent 1 are added to each tube. 3 ml. of Reagent 2 are rapidly added to duplicate or triplicate samples as the case may be. The tubes are immediately covered with glass bulbs and, after the contents are mixed, are placed simultaneously in a boiling water bath. The time required to add Reagent 2 to three tubes and place them in the water bath need not exceed 1 minute. After heating for 30 minutes the tubes are removed to a cold water bath in sets and in the same order in which they were placed in the boiling bath, so that each set of tubes is subjected to exactly 30 minutes heating. Reagents 3 and 4 are mixed in approximately equal portions and, when the tubes have

TABLE I

Titration Difference for Sorbitol and Mannitol with Varying Concentrations

Concentration per 5 ml.	Titration difference, 0.005 N thiosulfate	
	Sorbitol	Mannitol
<i>mg.</i>	<i>ml.</i>	<i>ml.</i>
0.1	0.92	0.93
0.3	2.63	2.73
0.5	4.36	4.17
0.7	5.83	4.96

cooled, 5 ml. of this mixed reagent are blown into each tube from a fast flowing 5 ml. pipette. The liberated iodine is titrated with 0.005 N thiosulfate, starch indicator being used near the end-point.

Heating Time and Factor Equivalents—In a method to be used for routine work it is expedient that the time required to complete an analysis be as short as possible. The length of time allowed for the reduction is of prime importance in this connection. Recoveries of known amounts of sugar alcohols in pure solution have been tried with various heating periods from 10 minutes to 1 hour. It was found that the shortest time of heating commensurate with the accuracy desired is 30 minutes. After this interval the reaction is fairly well completed and little error results from slight discrepancies in timing.

The relation between sorbitol concentration and titration difference is not linear and a curve must be prepared for the calculation of results. This is true for all of the alcohols investigated. Table I gives our values for sorbitol and mannitol in varying concentrations of pure solutions. It is a relatively simple matter for anyone wishing to make use of the method to develop curves for his own use.

Recovery of Sorbitol and Mannitol—Recovery of known amounts of sorbitol and mannitol ranged from 95 to 110 per cent. Treatment with HgSO_4 and BaCO_3 (*vide infra*) does not interfere with recoveries. From blood and urine 85 to 105 per cent of added sorbitol or mannitol has been recovered regularly.

Determination in Urine and Blood—The procedure given below has been used in following the excretion of sorbitol in urine and its concentration in blood after intravenous injection (1). Since glucose and other substances reduce the ferricyanide reagent, it is necessary to determine the sorbitol-reducing equivalent of the fluids before giving the sorbitol and to subtract these values from those found following its administration. The West-Scharles-Peterson (2) method of clarification has been used for blood and urine.

Urine—10 ml. of urine are added to 75 ml. of water in a 250 ml. Erlenmeyer flask and 15 ml. of HgSO_4 reagent (28 per cent HgSO_4 in 2 N H_2SO_4) are added; the mixture is neutralized with BaCO_3 (about 28 gm.) and filtered. 1 gm. of zinc dust per 15 ml. of filtrate is added to remove traces of mercury. After filtering again through a fine paper such as Whatman No. 42, the filtrate is diluted according to the concentration of sorbitol present and the reduction estimated as outlined.

Blood—5 ml. of blood are added to a flask containing 90 ml. of water. After laking is complete 5 ml. of HgSO_4 reagent are added and the mixture neutralized with about 9 gm. of BaCO_3 . Zinc dust is employed as in the case of urine filtrates. The final filtrate is diluted as necessary before analysis. The reducing equivalent of sorbitol and the other alcohols is changed by the presence of glucose in the blood. Consequently corrections must be made for this contingency.

We have accomplished this by determining the glucose equivalent in blood filtrates free of sugar alcohol and then determining

the reduction in another filtrate of the same blood after the addition of known amounts of a sugar alcohol. By simple calculation a factor for the compound in question is obtained. Large variations in the glucose content of blood samples under experimental conditions necessitate the determination of such factors concurrently with sugar alcohol estimations.

DISCUSSION

Potassium ferricyanide is not stable in strong alkali; a slow decomposition occurs with the production of ferric hydroxide. The ferricyanide reagent is thus prepared in aqueous solution. Sugar alcohols react in the cold with ferricyanide in alkaline solution and for this reason samples for analysis are placed in the water bath immediately following the addition of the alkali. Another point that must be considered is the reaction between the potassium iodide-zinc acetate mixture and glacial acetic acid. When these are mixed, iodine is slowly liberated and it is essential that this solution be prepared immediately before use. The end-point in the titration is well defined and little difficulty is encountered if good lighting is provided.

The method outlined has proved very satisfactory for biological work even in view of the limitations resulting from the fact that many substances in physiological solutions besides sugar alcohols react with the ferricyanide solution in the strongly alkaline medium employed. In blood filtrates, for instance, recoveries of added sorbitol were apparently very poor before it was ascertained that sorbitol exhibits less reduction in the presence than in the absence of glucose. The reason for this we believe is that glucose is attacked far more readily at the high pH by the ferricyanide reagent than is sorbitol. The result is that less ferricyanide is present after a short initial period of reduction to oxidize the sorbitol. It is, therefore, necessary in blood work to determine a factor for sorbitol in the presence of glucose. After this rather simple expedient was worked out, it was found that recoveries of added sorbitol from blood were sufficiently accurate for our purpose. Because the sugar alcohols do not reduce the ordinary copper reagents, it is possible to determine blood sugar in the presence of these compounds.

SUMMARY

A rapid method for the estimation of sugar alcohols is presented. 0.1 to 0.7 mg. in 5 ml. of solution may be determined in properly clarified filtrates of blood, urine, etc.

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ON THE METABOLISM OF SORBITOL AND MANNITOL*

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Following the demonstration (1) that sorbitol acts as an efficient diuretic in dogs, we have interested ourselves in a study of the metabolism in experimental animals of this sugar alcohol and one of its isomers, mannitol. A review of available literature concerning the metabolism of these compounds reveals considerable difference of opinion as to utilization, glycogen formation, etc., in animals. Variations in species response and in techniques employed may in part explain the discrepancies reported. The value of sorbitol in the diabetic diet is similarly controversial.

As early as 1883 Jaffe (2) demonstrated that after feeding mannitol to dogs large quantities can be recovered unchanged from the urine. Rosenfeld (3) observed no significant increase in liver glycogen in dogs after feeding mannitol. Following the administration of 100 gm. each of several carbohydrates dissolved in a glass of water to fasting colored males, Field (4) reported an average increase in blood sugar of 10 mg. per cent when mannitol was given compared to a 40 mg. per cent increase after the ingestion of glucose. Ariyama and Takahashi (5) reported mannitol to be far inferior to starch as a carbohydrate source for rats. It was also noted by Lafon (6) that in mice mannitol is utilized to a very slight extent or not at all and is toxic in sufficient quantity. However, it was reported that sorbitol is not toxic up to 30 per cent of the ration and that it seems to be completely metabolized. On the other hand Lecoq (7) observed that both mannitol and sorbitol are completely utilized by pigeons when they comprise 35 per cent, and the only carbohydrate source, of a lipid-rich ration. 66 per

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cent of either in the diet resulted in death of the animals. Voegtlin, Dunn, and Thompson (8) studied a number of carbohydrates and listed mannitol as slightly or doubtfully protective to rats previously given normal lethal doses of insulin.

Only two papers have come to our attention regarding the possibility of glycogen deposition in fasted rats following the administration of mannitol. Carr, Musser, Schmidt, and Krantz (9) fasted rats for 24 hours and then fed cacao-butter containing 33 per cent mannitol. After 80 hours on this diet these animals showed large increases in liver glycogen compared to controls fed cacao-butter only. Silberman and Lewis (10) administered 2 to 4 ml. of 15 per cent mannitol by stomach tube to rats fasted 24 hours and after absorption periods of 2, 3, 4, and 6 hours found no increase in liver glycogen over controls which had received like amounts of water. These authors stated that their results cannot be compared with those of Carr *et al.* because of the great dissimilarity in technique employed. We are unaware of any work substantiating or refuting these results.

Embden and Griesbach (11) reported that sorbitol solution perfused through livers from dogs previously phlorhizinized resulted in the formation of a mixture of fructose and glucose. However, lactic acid was formed when liver tissue from dogs fasted but not phlorhizinized was employed. Koike (12) found increased blood sugar and blood lactic acid following the intraperitoneal administration of sorbitol to rabbits, but Roche and Raybaud (13) stated that this alcohol is not transformed into liver glycogen by the fasting rabbit and that it is without effect on insulin hypoglycemia in these animals. Payne, Lawrence, and McCance (14) reported the failure of ingested sorbitol to increase the liver glycogen content of fasted rats. Thannhauser and Meyer (15) fasted dogs and then fed lean meat and glucose or lean meat and sorbitol for 3 days. The liver glycogen content of both groups showed about equal values and they stated that sorbitol is built into liver glycogen as readily as is glucose.

A number of authors (14, 16-18) are of the opinion that sorbitol has a place in the diabetic diet, while others (19-23) feel that it is to be avoided in such a diet.

Because of the controversial nature of the data and interpretations relative to the metabolism of sorbitol and mannitol in the

animal body, the writers have undertaken experiments in an attempt to clarify the situation.

Recovery from Urine of Sorbitol Given Intravenously—Normal female dogs were catheterized and then given 50 ml. of 50 per cent sorbitol intravenously over a period of 5 minutes. At varying intervals for 24 hours urine was collected by catheterization, or from the metabolism cages if voided, and kept in an ice box under toluene for sorbitol estimations. (Methods for the determination of sorbitol and other sugar alcohols are given in the preceding paper.) It is seen from the data in Table I that approximately 40 to 50 per cent of the injected sorbitol was recovered from the urine. Practically no excretion of sorbitol occurred after the first 24 hours. The remaining 50 or 60 per cent was apparently metabolized, as will be shown in a later discussion. Four such experiments were carried out on each of two dogs; representative data are given in Table I.

The figures in Table I are subject to some error and are to be taken as a close approximation only. With the method of determination employed dog urine exhibits considerable reduction (5 to 10 mg. per ml.) and consequently sorbitol determinations on urine in these experiments actually give total reduction which is not all attributable to the sorbitol present. Total reduction was determined on 24 hour urine samples from two dogs for 7 consecutive days. A range of 0.4 to 2.6 gm. reduction per day was found with an average of 1.4 gm. daily (average of both animals). Considering this amount of reduction from sources other than sorbitol during the above experiments, an average error in sorbitol recovery of about 12 per cent is introduced. The figures in the third column of Table I are thus high. The average per cent recovery in Table I is 44.7 and after the above correction is applied this figure becomes 39 per cent, which may be closer to the true value.

Clearance of Sorbitol from Blood Following Its Intravenous Injection—Normal dogs were given 50 ml. of 50 per cent sorbitol intravenously over a period of 5 minutes. Blood samples were withdrawn just previous to and at varying intervals following the injection. The samples were oxalated and subjected to sorbitol estimation at once. In a number of instances this work was carried out concurrently with the urine studies cited above. In Fig. 1 representative curves are presented for three dogs. Sor-

bitol was rapidly cleared from the blood following the high values attained a few minutes after the end of the injection. In all instances a peak of 400 to 700 mg. per cent was reached a few minutes after the injection and generally a value rather near the basal figure was attained within 2 or 3 hours. However, there is a

TABLE I

Recovery of Sorbitol from Dog Urine after Intravenous Injection of 50 Ml. of 50 Per Cent Sorbitol Solution

Dog No.	Recovered from urine in 24 hrs.	Recovery
	gm.	per cent
1	10.13	40.5
	12.14	48.5
3	9.53	38.1
	12.95	51.8

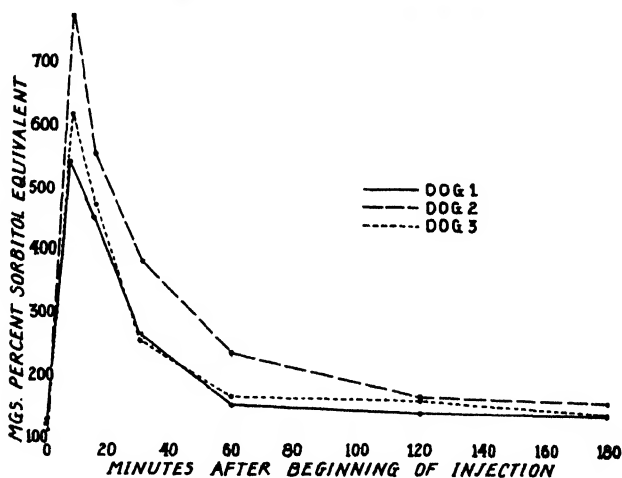


FIG. 1. Blood sorbitol curves following intravenous injection

distinct tendency for the curve, although flattened at 2 or 3 hours, to remain above the basal figure for several hours. This phenomenon has not yet been investigated. In eight such experiments on these three dogs the curves all fall within the limits of curves plotted in Fig. 1.

It is to be remembered that these curves are not true values for

sorbitol but represent total reduction including glucose, sorbitol, and undoubtedly other non-sugar reducing substances. It is felt that the validity of the data is little affected by this circumstance, as basal values were always determined. This figure was found to be quite constant; in eleven experiments in which basal blood reduction was determined with our method a range of from 118 to 146 mg. per cent was found, while in seven of these instances values between 130 and 140 mg. per cent were noted.

Blood Sugar Level Following Injection of Sorbitol and Mannitol—The reports in the literature on the conversion of sorbitol to glucose in experimental animals are controversial. Under certain conditions mannitol may lead to the formation of glucose, although little work has been reported on this point. Quantitative data and information regarding the speed of such reactions are lacking.

Normal dogs were given 50 ml. of 50 per cent sorbitol solution intravenously over a 5 minute period. Blood sugar was estimated on aliquots of the blood samples drawn for sorbitol studies, as described above. Clarification of the blood was accomplished by the method described by Steiner, Urban, and West (24) and the sugar was estimated by the Shaffer-Somogyi technique (25) with their Reagent 50. Mannitol and sorbitol do not reduce this reagent.

Fig. 2 indicates the nature of the true sugar curves. It will be seen that a peak of approximately double the basal value is reached about 30 minutes after the injection and that in 2 or 3 hours the basal figure is approached.

As mannitol is much less soluble (15 parts per 100 at 18°) than sorbitol, it was necessary to use relatively dilute solutions in order to compare the two compounds. In these experiments a dog was anesthetized with nembutal and given 150 ml. of 15 per cent mannitol intravenously over a period of 5 minutes. Blood samples were taken as previously and sugar estimations made at once. 3 days later the same dog was employed similarly except that 150 ml. of 15 per cent sorbitol were administered. The curves in Fig. 3 indicate that under these conditions sorbitol leads to an increase in blood sugar, while mannitol does not. The drop in the sugar curve after mannitol may be a result of blood dilution.

Formation of Glycogen from Sorbitol and from Mannitol in Fasted Rats—As the literature offers some confusion regarding the

possibility of conversion of sorbitol and mannitol into glycogen in experimental animals, we have administered these compounds to rats with the hope of clarifying the situation with regard to this species at least. Adult white rats, weighing from 150 to 250

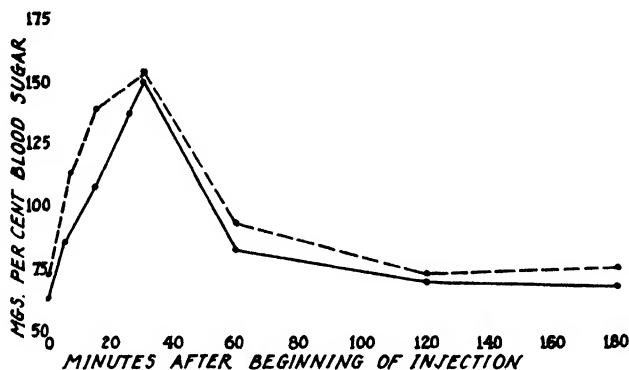


FIG. 2. Blood sugar curves for Dog 3 following 50 ml. of 50 per cent sorbitol given intravenously.

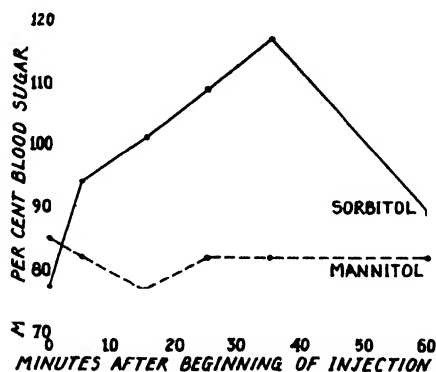


FIG. 3. Blood sugar curves for Dog 4 after the intravenous administration of 150 ml. of 15 per cent sorbitol or mannitol.

gm., from our stock colony were fasted for 40 hours and then given a solution of sorbitol or mannitol by stomach tube or by intraperitoneal injection. After various absorption periods the animals were decapitated, the livers removed and ground in a small meat grinder, and a sample immediately placed in a weighed tube

of alkali. With a little practice this entire operation can be completed in about 1 minute. The glycogen was determined by the Good-Kramer-Somogyi method (26). A number of the animals in each experiment received no supplement and acted as a control group.

TABLE II
Glycogen Storage in Livers of Fasted Rats (40 Hours) Following Administration of Sugar Alcohols

No. of rats	Substance	Method of administration	Total given	Dose volume	Solution	Interval between doses	Time killed after first dose	Per cent liver glycogen average
			ml.	ml.	per cent	hrs.	hrs.	
3	Sorbitol	Stomach tube	2	1	50	4	8	1.06
3	None		0				8	0.14
5	Sorbitol	Intraperitoneally	8	4	12.5	2	6	1.73
5	None		0				6	0.26
5	Mannitol	Stomach tube	4	2	20	2	5	0.33
5	None		0				5	0.33
5	Mannitol	Intraperitoneally	8	4	12.5	2	5	0.27
5	None		0				5	0.33

TABLE III
Glycogen Deposition in Livers of Fasted Rats (24 Hours) Subsequently Fed for 72 Hours

Eight animals were used in each instance.

Food consumption	Fed	Average weight loss	Average glycogen
gm.		gm.	per cent
13.4	Cacao-butter only	37	0.27
11.3	" + 33% mannitol	35	0.97
12.4	" + 33% sorbitol	43	0.79

Table II presents data from several experiments. It is apparent that sorbitol is readily converted into liver glycogen, while mannitol is not handled in the same manner under these experimental conditions.

Glycogen Storage in Previously Fasted Rats after Long Periods of Mannitol Feeding—Our results are in accord with those of Silberman and Lewis (10) that mannitol *per os* does not lead to glycogen deposition in fasted rats when only a few hours are allowed for absorption. However, the situation is apparently different when such animals are allowed to feed over long periods on mannitol and fat (Carr *et al.* (9)). We have further substantiated the findings of these authors using their technique, although our results show considerably less glycogen deposition.

Rats were fasted 24 hours and then fed cacao-butter containing 33 per cent mannitol or 33 per cent sorbitol *ad libitum* for a period of 72 hours. Controls were given cacao-butter only. At the end of this period the animals were sacrificed and their liver glycogen estimated, as previously described. The data in Table III indicate that the ingestion of either of these sugar alcohols leads to the deposition of liver glycogen under these conditions.

DISCUSSION

It was recently pointed out by Mirski *et al.* (27) that, when rats previously fed high protein diets were fasted, glyconeogenesis was highly intensified. This protein effect was also evident in animals which had been fasted 3 days, for, as explained by these workers, the rat organism uses up its store of carbohydrate during the first 24 hours of fasting and after that is living on its stock of protein and fat. We have noticed that on several occasions when our animals were fasted more than 48 hours liver glycogen values were found which were considerably above the usual 0.1 to 0.3 per cent for fasting rats. This we feel is explainable on the basis of the protein effect described by the above authors. Low figures for liver glycogen can be obtained regularly by fasting rats 40 hours.

As far as we are aware this paper presents the first data indicating that in the dog the intravenous injection of sorbitol results in an elevation of the blood sugar. We are also unaware of other attempts to study quantitatively the recovery of sorbitol from the urine or its clearance from the blood after its administration to

The writers wish to express their appreciation to Dr. H. C. Speel of the Atlas Powder Company for kindly supplying sorbitol

and mannitol for this work. They are indebted to Dr. Hance Haney for assistance in the dog experiments.

SUMMARY

1. The intravenous injection of sorbitol leads to an increased blood sugar in dogs. Mannitol does not exhibit this action.

2. Shortly after the injection of 50 ml. of 50 per cent sorbitol into dogs, blood clearance curves for this compound show a peak and a rapid decrease within the 1st hour. At 2 hours values close to the basal figure are reached but are maintained slightly elevated for several hours.

3. Under the same conditions about 40 to 50 per cent of the sorbitol can be recovered from the urine in 24 hours. After this time the excretion is negligible. The remainder of the sorbitol is apparently utilized.

4. When sorbitol is administered to fasted rats by stomach tube or by intraperitoneal injection, a deposition of liver glycogen occurs within 8 hours. Mannitol similarly administered does not lead to such a deposition.

5. When fasted rats are fed over a 72 hour period on mixtures of either sorbitol and cacao-butter or mannitol and cacao-butter, glycogen is deposited in their livers.

6. It is evident that sorbitol is much more readily converted into glucose and glycogen in the animal body than is mannitol.

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STUDIES IN PROTEIN METABOLISM

I. GENERAL CONSIDERATIONS IN THE APPLICATION OF ISOTOPES TO THE STUDY OF PROTEIN METABOLISM. THE NORMAL ABUNDANCE OF NITROGEN ISOTOPES IN AMINO ACIDS*

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(Received for publication, October 3, 1938)

In 1937 Urey and collaborators (1) reported¹ the concentration of the nitrogen isotope of atomic weight 15 (N^{15}) up to 2.5 per cent and this has since been increased to more than 15 per cent (2). Our aim has been to use this isotope as an indicator for the investigation of protein and amino acid metabolism in healthy animals on ordinary diets in a way similar to that in which deuterium had been used for the study of fat and sterol metabolism.

Two different types of isotopes, the radioactive and the stable, are available for studies of biological reactions. Work with radioactive isotopes is facilitated by the ease of analysis, and numerous experiments with inorganic compounds, particularly those of phosphorus, have been carried out with such material in various laboratories. The field of application of these products seems at present to be limited to the study of inorganic metabolism.

The intermediary metabolism of organic compounds can be studied by using isotopes of only those elements which constitute organic matter (C, H, O, N). No radioactive isotopes of these elements are known with a half life time long enough to permit their use for experiments involving synthesis, administration, and isolation from the animal organism. This type of work requires the use of stable isotopes, which are obtained by lab-

* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

¹ Prior to publishing his results Dr. Urey kindly supplied us with samples of isotopic ammonia for biological work.

oratory fractionation of the natural elements. They involve more complex analytical methods.

The general considerations outlined in the beginning of the work with deuterium (3) apply to some extent also to the studies with N^{15} . The abundance of this isotope in ordinary nitrogen is 0.368 per cent, and as far as is known this concentration is about the same in all nitrogen samples, independent of their source. We have carried out determinations of this isotope in various natural amino acids, using methods described in Paper II of this series, and have found the same concentration of N^{15} as in the nitrogen of the air. The equal distribution of the isotope in air and in inorganic and organic compounds can be taken as an indication that animals or plants in the synthesis and catabolism of nitrogenous compounds do not appreciably fractionate the nitrogen isotopes (N^{14} and N^{15}) but treat the two indiscriminately. A normal monoamino acid is a mixture of molecules, 99.63 per cent of which contains only N^{14} and 0.37 per cent of which contains only N^{15} . If a physiological compound be administered in which the concentration of N^{15} is slightly increased (i.e. it contains more molecules with N^{15} than normal), this cannot constitute a substance foreign to the animal, as the cells are normally accustomed to both species of molecules.

The mass spectrometric procedure described in Paper II of this series is an unusually sensitive micromethod. An increase of 0.003 per cent N^{15} above the normal 0.368 can be detected in less than 1 mg. of nitrogen. The sensitivity thus is of the same order as that used in routine deuterium determinations, and the total amount of material to be used is much smaller. A very small amount of glycine, the nitrogen of which contains only a 5 per cent excess of N^{15} above the normal concentration, can be mixed *in vitro* or *in vivo* with several hundred times its weight of ordinary glycine before the isotope tracer will be lost by the analytical method. Experience with deuterium has shown that when compounds with only a moderate isotope content are fed, it is almost always possible to follow the isotope in the animal in spite of the great chance that the administered deuterium might be diluted with ordinary hydrogen in the body, which contains a relatively enormous number of hydrogen atoms. The chance for dilution of N^{15} with ordinary nitrogen is much smaller, as the

living cell contains only one-fiftieth as many nitrogen atoms as hydrogen atoms. It is thus possible in metabolism work with nitrogen to use not only lower isotope concentrations, but also smaller quantities of isotope than were used in work with deuterium.

Deuterium has been employed as an indicator for water metabolism and as a label in organic compounds to study their transportation and transformations. There is no doubt that N^{15} can be employed similarly for studying the fate of the nitrogen given to an animal in organic or inorganic compounds. It will be shown in Paper III of this series that the carbon-nitrogen linkage of amino and guanido compounds is relatively stable *in vitro* and it is highly probable that the same is true for these compounds when present in the animal. A loss of isotopic nitrogen from an amino acid in the animal has to be expected only if chemical reactions occur at the amino group (*e.g.* deamination). The isotope can thus most probably be used as an indicator for the study of the *transportation* of such nitrogenous compounds in the animal.

It is more difficult to predict to what extent N^{15} can be employed for the studies of the *interconversion* of nitrogenous compounds. The physiologist in studying substances containing only C, H, and O is interested mainly in the fate of the carbon. The conversion of carbohydrate into fat means by definition the utilization of the carbon from the sugar for building up fatty acids. The fate of the hydrogen or oxygen atoms in this process is of minor interest. In work with nitrogenous compounds, however, the metabolic fate of both the carbon chain and the nitrogen atoms has to be considered. If one amino acid is converted into another, either the carbon chain or the amino nitrogen, or both, may conceivably be utilized in the process.

In previous publications, it has been shown that carbon-bound deuterium could in many cases be used as an indicator for the fate of carbon chains, and preliminary experiments (4) have indicated that this isotope might also be employed for studies on the amino acids. It is hoped that the use of two independent isotopic labels (D and N^{15}) in the same amino acid molecule may reveal a more complete picture of its metabolism. Amino acids containing both isotopes have already been synthesized and

tested biologically in exploratory experiments. The results encourage us to continue work with such compounds.²

In work with deuterium the isotope content of a compound is usually given as the excess over the normal concentration, which is 0.02 atom per cent. Monodeuterovaleric acid, $C_5H_7DO_2$, contains 10.00 atom per cent deuterium (1 deuterium atom in a total of 10 hydrogen atoms), but has an excess above normal of only 9.98 atom per cent, as the normal acid contains 0.02 per cent of deuterio compounds. The difference is in most cases so small that it may be neglected. In work with the nitrogen isotope, however, the normal concentration of N^{15} (0.368 per cent) is significant and has to be taken into consideration. In subsequent papers biological work will be described which was carried out with compounds, the nitrogen of which contained as little as 0.59 per cent N^{15} , or an excess of 0.22 per cent above normal. This excess alone characterizes the compound and has to be considered for calculations in chemical or biological work. We propose to characterize the isotopic compounds by their *excess N^{15} content* rather than by their absolute N^{15} concentration. The advantage of this will be apparent from the following example. Suppose an animal is given a glycine preparation, the nitrogen of which contains an absolute amount of 1.000 per cent N^{15} and mixes in the organs with normal glycine. From the animal there is then in turn isolated a glycine preparation, the nitrogen of which contains an absolute amount of 0.684 per cent N^{15} . The glycine fed contained 0.632 atom per cent excess (*i.e.* 1.000 - 0.368), and the material isolated contained 0.316 (*i.e.* 0.684 - 0.368) atom per cent. The glycine was, therefore, diluted by the same amount of normal glycine from the animal body. We shall thus describe a glycine, the nitrogen of which contains 1.000 per cent N^{15} as glycine (0.632 atom per cent N^{15} excess), the word "excess" indicating an excess above the normal abundance.

The term "atom per cent N^{15} " is in conformity with the nomenclature used in deuterium studies. "Valeric acid containing 10 atom per cent deuterium" means that 10 per cent of all the hydrogen atoms in the compound is present in the form of deuterium,

² The carbon isotope, if available, would supply a tool even more valuable for such purposes than deuterium.

and "glycine containing 0.632 atom per cent N^{15} excess" means that the nitrogen of the compound contains 0.632 per cent more N^{15} atoms than normal glycine.

EXPERIMENTAL

The amino acids listed in Table I are all natural compounds, isolated from proteins. The isotope concentration in the nitrogen of these substances was determined by the methods described in Paper II of this series.

TABLE I

Concentration of Nitrogen Isotope N^{15} in Natural Amino Acids

The values give the excess above the concentration of N^{15} in air (0.368 per cent).

Substance	N^{15} content atom per cent excess
Casein.....	0.004
Glycine.....	0.002
Tyrosine.....	0.000
Leucine.....	0.002
Proline.....	0.006
Glutamic acid.	0.004
Lysine.....	0.003
Histidine.....	0.000
Cystine.....	0.003
Arginine.....	0.008
	0.010
	0.007
	0.008

The values give the difference between the N^{15} concentration in the nitrogen of the compound and that of air.

DISCUSSION

It cannot yet be decided whether the slightly higher values found in the analyses of the amino acids and especially those of the four different arginine samples, 0.007 to 0.010 atom per cent excess, demonstrate a real increase in the N^{15} concentration, or are due to error. It is likely that in some chemical or biological reactions minute fractionation of the nitrogen isotopes occurs,

such as Brewer (5) has observed for the potassium isotopes in heart muscle and bone marrow. Urey has made use of such very small fractionations to concentrate the nitrogen isotope, by repeating the reactions thousands of times.

Whether the differences found in Table I are real or not, they are too small to interfere with the application of the nitrogen isotope as a label. As will be shown in subsequent papers, the feeding of isotopic physiological compounds may lead to the formation of other nitrogenous compounds with an isotope content far above the differences found in ordinary amino acids.

SUMMARY

1. The application of the stable isotope of nitrogen (N^{14}) for the study of protein metabolism is discussed.

2. The N^{15} content of casein and of nine different amino acids has been determined. The nitrogen liberated from these compounds has the same isotopic composition as the nitrogen from the air.

The authors are highly indebted to Dr. Harold C. Urey for the valuable gift of many samples of isotopic nitrogen, as well as for his continued interest, help, and advice in the course of our work with this isotope. They, furthermore, wish to express their gratitude to Dr. Hans T. Clarke for many valuable suggestions and for his continued encouragement and support during all the phases of the work.

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STUDIES IN PROTEIN METABOLISM

II. THE DETERMINATION OF NITROGEN ISOTOPES IN ORGANIC COMPOUNDS*

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The only practicable method for the precise determination of the isotopic composition of an element other than hydrogen involves the use of a mass spectrometer.

The determination of the isotopic composition of an element by measuring the atomic weight of the mixture is time-consuming, requires large samples, and is not sensitive to small variations in the isotopic composition. It is thus not applicable to biological work, in which isotopes are employed as tracers in organic molecules. High sensitivity and accuracy of the analytical procedure are required.

The mass spectrometer disperses by the action of an electric and magnetic field a beam of ions into a spectrum dependent on their masses, in analogy to the optical spectrometer which disperses quanta of light into a frequency spectrum. By measuring in an optical apparatus the ratio of the intensities of peaks present at different regions of the spectrum, the relative abundance of quanta of different frequencies can be determined. In the mass spectrometer the relative abundance of particles of different masses is determined. If applied to one element, the relative amounts of the various isotopic species can be determined directly by this procedure. In the case of nitrogen, the concentration of molecules $N^{14}N^{14}$ and $N^{14}N^{15}$ are compared, and this can be carried out with a precision of 0.003 per cent N^{15} , corresponding to 0.00003 atomic weight unit.

* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

This high sensitivity and accuracy are accompanied by many other desirable features. Only small samples of gas are necessary (as little as 0.5 cc. of nitrogen gas), and most impurities (except O_2 , which will be discussed later) are without effect on the analysis, as they have masses different from that of the nitrogen molecules. The procedure is rapid and applicable to routine.

The isotope analysis of the nitrogen in an organic compound is carried out with nitrogen gas liberated from it. The principal steps of the procedure are (1) liberation of the nitrogen in the form of ammonia by the Kjeldahl procedure, (2) conversion of the ammonia into gaseous nitrogen by alkaline hypobromite, (3) admission of the gas sample to the vacuum tube containing the spectrometer, (4) measurement of the isotopic composition of the sample.

EXPERIMENTAL

Liberation of Nitrogen As Ammonia by Kjeldahl Procedure—An amount of material corresponding to 0.5 to 2 mg. of nitrogen is treated in a micro-Kjeldahl flask with H_2SO_4 . Neither hydrogen peroxide nor other peroxides should be added, as they may give rise to the formation of molecular oxygen in the later stages of the procedure. The ammonia is distilled in an ordinary micro-Kjeldahl apparatus into dilute acid, and back titration gives the total amount of nitrogen present in the compound. After titration, the solution is made acid again and brought to a volume of 1 to 3 cc.

Conversion of Ammonia into Nitrogen—The reaction is carried



out in the evacuated apparatus shown in Fig. 1.

The solution containing the ammonium salt is placed in *A*, and an excess (about 5 cc.) of a strongly alkaline hypobromite solution placed in *B*. A dry ice bath is placed around the trap *C*, and the apparatus evacuated by a diffusion pump. The air dissolved in the solutions in *A* and *B* is removed during this evacuation. After about 2 minutes of pumping, the stop-cock between the system and the pump is closed and the vacuum tested.

The vessel *B* is rotated on the ground joint so that the hypobromite solution flows into *A*. The evolved nitrogen is pumped by

the Toepler pump *F* into tube *E*. This is closed at the upper end by the capillary *G* (the "break seal"), the use of which is described later. When all the gas is transferred to tube *E*, it is sealed off from the system. The sample is now ready to be connected with the inlet tube of the mass spectrometer.

Admission of Gas to Mass Spectrometer—The tube *E* with the break seal *G* is attached to the glass system shown in Fig. 2, which is connected with the mass spectrometer. It consists of (1) the very fine capillary *A* through which the gases enter the vacuum

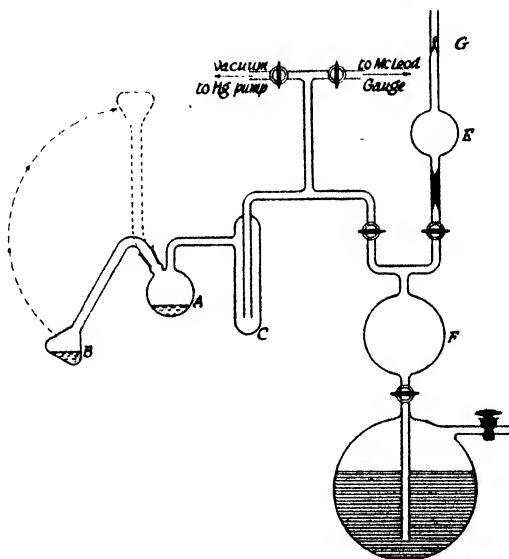


FIG. 1. Apparatus for the liberation of nitrogen

tube which holds the mass spectrometer, (2) the U-trap *B* which is immersed in liquid nitrogen to freeze out impurities, (3) the stop-cock *C* which isolates the mass spectrometer when air is admitted to the remainder of the glass system, (4) the open end manometer *D*, (5) the automatic Toepler pump *F* employed to pump very small gas samples from the right part of the system to the left which holds the capillary, and (6) the five stop-cocks 1 to 5 to which the tubes *E* containing the gas samples are sealed. Before they are attached to the system, an iron rod *H*, 5 mm. in diameter

and 15 mm. long, is carefully placed on top of the capillary *G*. The flask *J* contains normal nitrogen for testing the apparatus by measuring the normal abundance of N^{15} .

The glass system is evacuated by diffusion pump through stop-cock *K*. After this is closed, 20 mm. pressure of normal nitrogen are admitted from *J*. The isotope ratio of the normal nitrogen sample is now determined as described later and if the correct value of 135 is obtained, the unknown samples attached to stop-cocks 1 to 5 are admitted in the following manner: After the normal nitrogen is evacuated through *K*, this is closed and the break seal *G* is

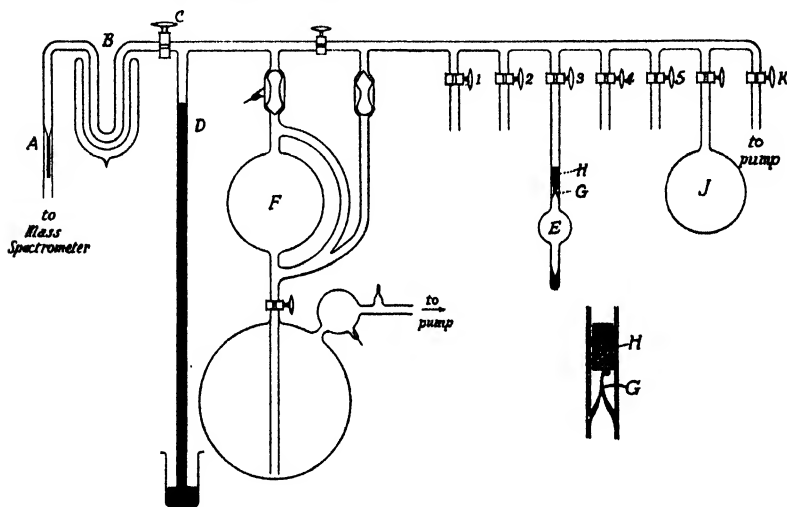


FIG. 2. Inlet to mass spectrometer

broken by lifting and releasing the iron rod *H* by means of a magnet handled from outside. After *G* is broken, the gas pressure behind the capillary leak *A* is adjusted to 20 mm., the Toepler pump *F* being used. The isotope ratio of the unknown sample is now determined as described later. After at least three measurements on the gas sample, it is pumped out through *K*. The apparatus is now ready for the next analysis, and another sample may be admitted.

Measurement of Isotopic Composition—The particular mass spectrometer which we have constructed is modeled after the one de-

scribed by Bleakney.¹ A simplified schematic diagram is shown in Fig. 3. The metal apparatus is placed in a glass tube which is continuously evacuated by a large diffusion pump. The heated filament *B* emits electrons which are drawn to plate *C* by a positive potential.² About half of these pass through the very narrow slit in plate *C* to give a thin ribbon of electrons *D*. After passing through the slit in *C* the ribbon of electrons passes between plates *E* and *F*. In this region the electrons strike and ionize the molecules of the gas which have been admitted into the spectrometer tube through the very fine capillary *A* (shown in Fig. 2). Those electrons which do not strike a gas molecule pass through the slit in the box *G* and strike plate *H*, where they are absorbed and conducted back to the filament *B*. The number of electrons which

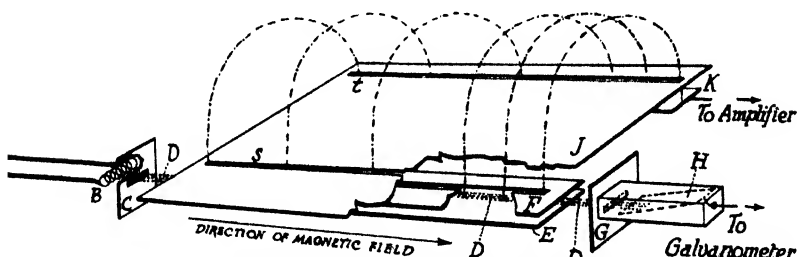


FIG. 3. Inner part of mass spectrometer

strike plate *H* is measured by a galvanometer in the return circuit. The total ionization in the region between plates *E* and *F* is proportional to the electron flow; *i.e.*, the current through the galvanometer. This is kept constant by controlling the temperature of the emitting filament *B*.

In the region between plates *E* and *F* the electrons create a ribbon of positive ions coincident in space with the electron beam. When they strike a molecule $N^{14}N^{14}$, they form a singly charged ion of mass 28, while when they strike an $N^{14}N^{16}$ molecule, they form one of mass 29. These ions are drawn out of the generating

¹ Bleakney, W. M., *Physic. Rev.*, **34**, 157 (1929); **40**, 496 (1932).

² Additional plates present in the instrument are omitted from the diagram.

region by a small negative potential on plate *F*, pass through the slit in this plate, and are then accelerated by a negative potential *V* on plate *J* which is under the control of the operator. The accelerated ions pass through a narrow slit *s* in plate *J*. The whole spectrometer is immersed in a magnetic field parallel to the direction of the electron beam and of constant intensity *h*. Under these conditions the path of the ions is a circle. The radius of the orbit is given by

$$r^2 = 2 \frac{mV}{eh^2} \quad (1)$$

where *m* is the mass of the ion, *e* is the charge of the ion, *V* is the negative potential on plate *J*, and *h* is the strength of the magnetic field. 8 cm. from the slit *s* in plate *J* is another slit *t*, behind which is a highly insulated collector plate, *K*. The only ions which can reach plate *K* are those which have an orbital radius of 4 cm. Since *V* is under the control of the operator, he can cause an ion of any mass to reach plate *K*. In practice all the quantities of Equation 1 are constant, with the exception of *V* and *m*. Equation 1 therefore reduces to

$$mV = \text{constant} \quad (2)$$

The constant for our spectrometer when *m* is expressed as the molecular or atomic weight and *V* in volts is about 1800. When *V* is set at 100 volts, particles of mass 18 can reach plate *K*, while to direct particles of mass 19 to it, *V* must be set at 94.7 volts. The ion current reaching plate *K* is proportional to the number of ions striking it, and, therefore, a measurement of this current directly determines the number of such ions present.

The current reaching plate *K* passes through a resistance of 2.4×10^9 ohms, and the voltage developed across it is measured by a very sensitive vacuum tube voltmeter. During the analysis of a normal nitrogen sample a current of the order of 10^{-11} ampere (6×10^7 ions per second) reaches plate *K* when the ions of mass 29 ($\text{N}^{14}\text{N}^{15}$) are collected, while a current 135 times greater reaches it when ions of mass 28 ($\text{N}^{14}\text{N}^{14}$) are being collected. The vacuum tube voltmeter is sensitive to 10^{-5} volt, which corresponds to an ion current of 10^{-14} ampere.

The mass spectrometer can be employed to determine the iso-

topic composition of any gas. It will determine the abundance ratios of the various molecules present in nitrogen ($N^{14}N^{14}$, $N^{14}N^{15}$, $N^{15}N^{15}$), hydrogen chloride (HCl^{35} , HCl^{37} , DCl^{35} , DCl^{37}), carbon dioxide ($C^{12}O^{16}O^{16}$, $C^{12}O^{16}O^{18}$, $C^{12}O^{18}O^{18}$, $C^{13}O^{16}O^{16}$, $C^{13}O^{16}O^{18}$, $C^{13}O^{18}O^{18}$), etc.

Our spectrometer covers the range from mass 16 to about mass 75. In Fig. 4 typical curves of normal tank nitrogen are given in

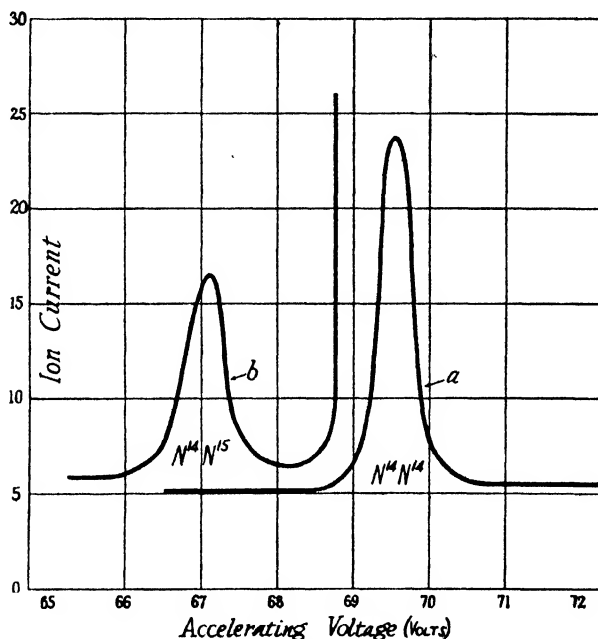


FIG. 4. Mass spectrum of normal nitrogen

which the ion current is plotted against the accelerating voltage (or mass). Curve a was obtained with the amplifier set at 1/1000 of its maximal sensitivity. The peak obtained at 69.5 volts (mass 28) is due to the ions $N^{14}N^{14}$. No ion peak is noticeable on this curve at mass 29 ($N^{14}N^{15}$), as its intensity is only 1/135 of that given by mass 28. If the sensitivity of the amplifier is increased 100-fold (by changing to one-tenth its maximal sensitivity), one obtains Curve b. The peak due to mass 29 (67.1 volts), as

well as the beginning of the peak due to mass 28, is observable. The ratio of the intensities of the ion currents (abundance ratio of the molecules $N^{14}N^{14}:N^{14}N^{15}$) can be converted into atom per cent of N^{15} by the formula

$$P = \frac{100}{2R + 1} \quad (3)$$

where P is the atom per cent of N^{15} and R the ratio just mentioned. When R is 135, then P is 0.368 atom per cent.

Effects of Impurities—Before analysis is begun, the spectrum given by the evacuated instrument is examined. No peaks except that given by water (mass 18) should appear. The presence of other peaks is evidence of impurities in the vacuum tube. They are removed by heating the evacuated tube.

The gas sample to be analyzed is passed, before it enters the vacuum, through a trap cooled with liquid nitrogen, which removes all condensable impurities. The only troublesome impurity which can pass the trap is air, which may have entered through a leak in any of the glass vessels in which the gas has been handled. The nitrogen of the air would dilute the sample. The presence of air immediately becomes apparent during the analysis, since the oxygen sharply lowers the electron emission of the filament. The presence of a peak of mass 32 (oxygen molecules) and mass 40 (argon) in the spectrum is further proof. Samples showing these peaks are discarded. In all analyses a search for peaks other than those of masses 28 and 29 is routinely carried out.

Sensitivity of Instrument—On the basis of over forty analyses of ordinary nitrogen performed at least in triplicate as well as more than 100 analyses of nitrogen samples obtained from organic compounds, most of which had an excess of N^{15} , we conservatively estimate the error of a determination of the atom per cent N^{15} to be of the order of 1 per cent.

Our instrument has given for the concentration of N^{15} in ordinary (tank) nitrogen a value of 0.368 per cent with an average deviation of 0.003 per cent. This value is not corrected for the fractionation which occurs during the passage of the isotope mixture through the fine capillary A (Fig. 2). This small error does not affect our work, since it is a constant of our instrument and enters all analyses in the same fashion.

Error of Analysis of Isotopes in Organic Compounds

The total error of the analyses of nitrogen from organic material is slightly higher than the sensitivity of the mass spectrometer. This is due to a slight dilution of the nitrogen liberated from the compounds with the nitrogen of ammonia present as impurities in the reagents employed (H_2SO_4 , NaOH , etc.). This error becomes higher when the amount of nitrogen taken for analysis is small. In our work sufficient nitrogen was taken for analysis to keep the dilution below 3 per cent. An error of this kind does not interfere at all with biological work.

SUMMARY

The determination of the nitrogen isotope N^{15} in organic compounds with a mass spectrometer is described.

The authors wish to express their gratitude to Dr. Harold C. Urey and to Dr. M. Fox for their advice during the construction of the mass spectrometer.

STUDIES IN PROTEIN METABOLISM

III. SYNTHESIS OF AMINO ACIDS CONTAINING ISOTOPIC NITROGEN*

BY RUDOLF SCHOENHEIMER AND S. RATNER

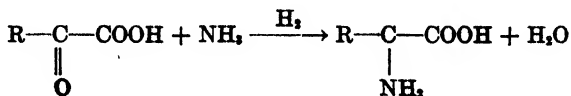
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(Received for publication, October 3, 1938)

The starting material for amino acid synthesis was ammonium salt (chloride or nitrate) containing isotopic nitrogen. The salt was kindly supplied to us by Dr. Harold C. Urey (1). Various ammonia preparations were used, the nitrogen of which contained an excess of from 0.22 to 6.65 per cent N^{15} above normal. For synthetic work with the isotope the usual laboratory procedures could not be employed. All the known methods had been worked out with the aim of obtaining good yields when calculated for the carbon chain, while the recovery of the nitrogen was neglected. In work with the isotope the nitrogen is always of incomparably higher value than that of even the most complex carbon chain. The synthesis must be directed in such a way that all nitrogen employed is recovered either in the form of amino acid or as ammonia. Reactions which lead to nitrogenous side products must be avoided. The common synthesis of amino acids by treating the α -bromo acid with a large excess of ammonia (50 to 70 moles) (2) is not practicable for our purposes.

We have modified two known procedures: A, the catalytic hydrogenation of α -keto acids in the presence of ammonia as described by Knoop and Oesterlin (3); B, the coupling of α -bromo esters with potassium phthalimide as described by Gabriel and Kroseberg (4).

Procedure A—



* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

By the general method of Knoop and Oesterlin, the reduction of the keto acid was carried out in 50 per cent alcohol with palladium. For the reduction of monocarboxylic keto acids 2 equivalents of ammonia must be used, 1 for the carboxyl, and 1 for the keto group; for the dicarboxylic keto acids 3 moles of ammonia are necessary. After the reaction, the ammonia not present as amino nitrogen was recovered quantitatively by distillation; with the dicarboxylic acids, the addition of barium hydroxide is necessary. No nitrogenous side products were observed in the reaction, and the amino acids obtained were in all cases sufficiently pure without recrystallization. The yields, even when calculated on the basis of the keto acid, were good except in the preparation of alanine and aspartic acid, in which losses were most probably due to the formation of some hydroxy acid. Even in these preparations no appreciable amount of isotopic nitrogen was lost.

The following racemic amino acids containing isotopic nitrogen have been prepared in this way: alanine, norleucine, phenylalanine, tyrosine, glutamic acid, and aspartic acid.

Procedure B—The phthalimide procedure has been modified in such a way that it fulfils the same requirements as Procedure A; namely, no loss of the isotopic nitrogen. It was employed for the synthesis of amino acids which could not easily be obtained by Procedure A, such as glycine, deuteroleucine, and lysine.¹

The first step, the preparation of isotopic phthalimide, involves no loss of ammonia. In contrast to earlier workers (5), we have used only 1 mole of ammonia for 1 mole of phthalic acid (instead of phthalic anhydride) without a decrease in the yield of phthalimide. Small amounts of nitrogen present in the mother liquors of the phthalimide or potassium phthalimide can be recovered quantitatively in the form of ammonia after alkaline hydrolysis of the imide remaining in solution.

The coupling of the bromo ester with potassium phthalimide requires a temperature of 150° in the preparation of glycine, but higher temperatures are necessary in the case of amino acids of higher molecular weight (up to 200°). At this temperature some hydrobromic acid is split off from the bromo ester, giving rise to free phthalimide. The nitrogen which thus is not used for the formation of amino acids can be recovered by alkaline treatment

¹ The preparation of isotopic lysine will be described later.

of the mother liquors. In some cases the addition of cupric oxide catalyzed the coupling reaction, so that the temperature could be decreased, and a better yield secured.

According to Gabriel the hydrolysis of the phthalimino ester is carried out either with concentrated hydrochloric acid at 180° in a closed tube, or by a two-step hydrolysis, first with 1 mole of alkali, then with concentrated hydrochloric acid at 100° . The first procedure involves the danger of breakage of the pressure tube, and the second method, loss in the isolation of the intermediate free phthalimino acid. The one-step hydrolysis by refluxing with a mixture of acetic acid and hydrochloric acid, described by Ratner and Clarke (6) for the preparation of thiazolidine, has also proved to be valuable for our purpose. In the case of glycine the yield of amino acid in this last step is 98 per cent.

Procedure C. Preparation of Amino Acids Containing Both Isotopes D and N¹⁵—For reasons discussed in Paper I of this series (7) methods had to be devised for the preparation of amino acids which contain carbon-bound deuterium in addition to N¹⁵. We have prepared *dl*-leucine with both isotopes, by a method which is also applicable to other amino acids.

Kinney and Adams (8) have described the preparation of leucine- β, γ - d_2 by hydrogenating isobutenal diethyl acetal with deuterium gas. This procedure could not be employed for our purpose, as the introduction of nitrogen into the starting material as well as the subsequent steps would lead to considerable loss of the nitrogen isotope.

A procedure has recently been described for the preparation of fatty acids containing stably bound deuterium (9), by treatment of ordinary fatty acids with heavy water at high temperature in the presence of active platinum and alkali. This method cannot be directly applied to amino acids. For reasons still obscure only very small amounts of stable deuterium were introduced into amino acids when treated under the same conditions. We have, therefore, prepared the isotopic leucine by first introducing deuterium into the corresponding fatty acid, isocaproic acid. This was brominated in the α position and the bromo ester was treated with potassium phthalimide (N¹⁵) according to Procedure B. Despite the drastic conditions to which the deuterioisocaproic acid was subjected in the course of the preparation, the resulting

leucine contained 3.87 atom per cent deuterium in addition to 6.49 atom per cent N^{15} excess, a new indication for the high stability of the carbon-bound hydrogen in this amino acid (10).

EXPERIMENTAL

Procedure A. Hydrogenation of α -Keto Acids in Presence of Ammonia According to Knoop and Oesterlin—Each step of the procedure has been devised with the aim of avoiding loss of the isotopic nitrogen and of recovering all excess in the form of ammonia. Care had to be taken to avoid dilution of the isotopic ammonia with ordinary ammonia. The nitrogen of the amino acid obtained had in all cases practically the same isotopic concentration as that of the ammonia with which the reaction was started, indicating that no dilution of the isotope had occurred.

In order to obtain good yields of amino acids it is necessary to use a large quantity of active palladium (3 to 4 gm. for the reduction of 0.05 mole of keto acid). With smaller amounts, a slower reduction and a presumably increased production of hydroxy acid occur. As the temperature of the reaction should not exceed 20° , during the warm season the reaction bottle was cooled by wrapping it with a wet cloth.

All distillations of ammonia were carried out in a stream of inert gas to avoid bumping and to insure a smooth distillation. As elementary nitrogen does not exchange with ammonia (11) under the conditions of our experiments, nitrogen gas from a cylinder was used for the purpose.

Distillation of Isotopic Ammonia—The isotopic ammonia is generated from ammonium nitrate or chloride by means of strong alkali and distilled directly into the hydrogenation vessel where it is absorbed by 95 per cent alcohol cooled with solid CO_2 . The 500 cc. hydrogenation flask *A* (Fig. 1) has an interchangeable glass joint *B* and a long side arm *C*. For the hydrogenation as described below, *B* is closed with a glass stopper and the flask is shaken in a special apparatus in which the side arm *C* serves as the axis.

The ammonia is liberated in the 100 cc. modified Claisen flask *D* containing 40 cc. of 10 *N* NaOH. The arm *H* is fitted with a condenser to prevent water from distilling into *A*. The other arm of *D* is equipped with a dropping funnel *E* and a gas inlet tube *F*.

The hydrogenation flask *A* contains 3 to 4 gm. of palladium black suspended in 95 per cent alcohol. The distilling tube *G* which is fitted into the flask *A* at the glass joint *B* and into flask *D* at the joint *H*, extends under the liquid in *A*. The arm *C* is connected with the gas wash bottle *I* containing dilute H_2SO_4 to recover any ammonia not absorbed in *A*. A slow stream of nitrogen gas is bubbled through the system by means of tube *F* so as to minimize pressure differences during the absorption and to prevent the sucking back of liquids in *A* and *I*.

Flask *A* is cooled with solid carbon dioxide and flask *D* gently heated to boiling with a microburner. A concentrated solution of the ammonium salt containing the isotopic nitrogen is slowly

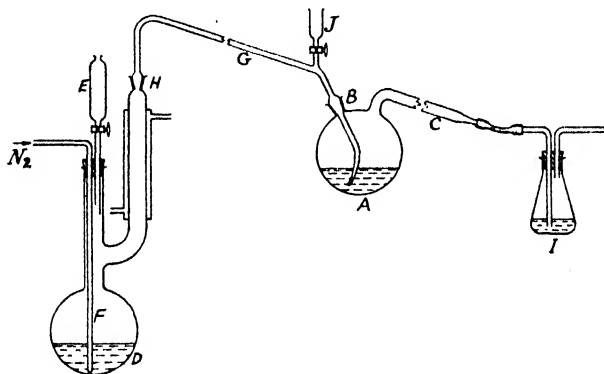


FIG. 1. Apparatus for distillation of isotopic ammonia

added through the dropping funnel *E*. Heating is continued until all NH_3 has been driven over into *A*. The arm *G* is then removed and the tip rapidly rinsed with a few cc. of alcohol through *J*. The α -keto acid in 70 cc. of H_2O per 0.1 mole is rapidly added to *A*, which is cooled with dry ice. *B* is closed with a glass stopper and the flask connected with the hydrogenation apparatus.

Hydrogenation—The hydrogenation apparatus (Fig. 2) is designed to permit the maintenance of a sufficient gas pressure (slightly below atmospheric) for rapid hydrogenation without the use of any liquid in the hydrogen reservoir. It consists of the 5 liter gas storage bulb *B* and a manometer *C*. The hydrogen tank is connected at *D* and a vacuum pump at the 3-way stop-cock *E*.

Bulb *B* and the glass tubes between *E* and *D* are evacuated and filled with hydrogen. *D* is closed, and *E* is connected through a rubber tube with the hydrogenation flask *A* which is still cooled with solid CO_2 . The air in *A* is then displaced by a stream of hydrogen which finally passes through a wash bottle containing H_2SO_4 to absorb any ammonia carried over with the hydrogen. The flask *A* is quickly stoppered with a glass stopper, *D* closed, and the cooling of *A* discontinued. After the reaction mixture has attained room temperature, shaking is begun. The hydrogen

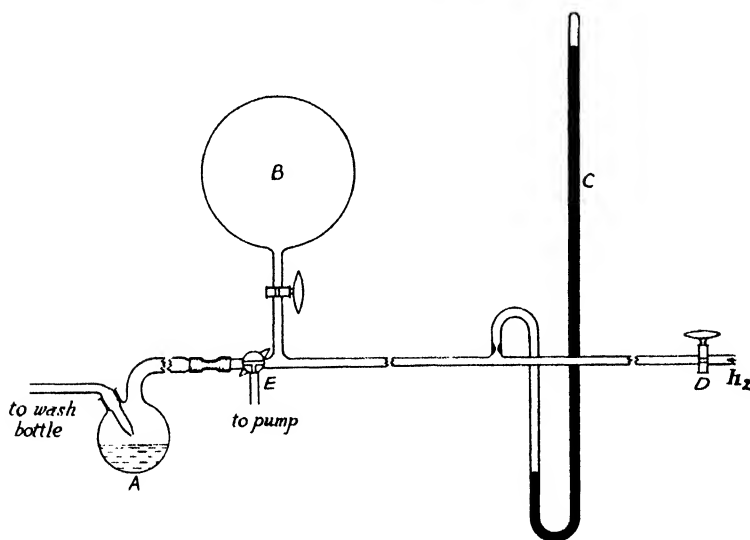


FIG. 2. Apparatus for hydrogenation of keto acids

uptake can be calculated at any time from the change of pressure in the system, the volume of which is known. During the reaction the pressure is increased from time to time to just below atmospheric by adding hydrogen through *D*.

Recovery of Excess Ammonia—After hydrogenation is completed, all the excess ammonia is removed by distillation before isolation of the amino acid. The reaction flask *A* is again cooled with dry ice and connected with a condenser *B*, an adapter *C*, and the acid trap *D* (Fig. 3). A side arm *E* is added which provides for the addition of alcohol and the entrance of nitrogen gas. The re-

action flask is heated on a steam bath and alcoholic ammonia distilled over into the acid trap *D* in a stream of nitrogen until the distillate is neutral. Several additions of alcohol are necessary. Enough hot water is added to dissolve the amino acid and the catalyst is filtered off. The amino acid is isolated from the filtrate by appropriate means.

In the preparation of the monoaminodicarboxylic acids it is necessary to add a fixed base for complete recovery of ammonia. If the amino acid can be heated in the presence of alkali, as is the case with aspartic acid, 2 equivalents of $\text{Ba}(\text{OH})_2$ are added to the hydrogenation flask and the ammonia distilled off as de-

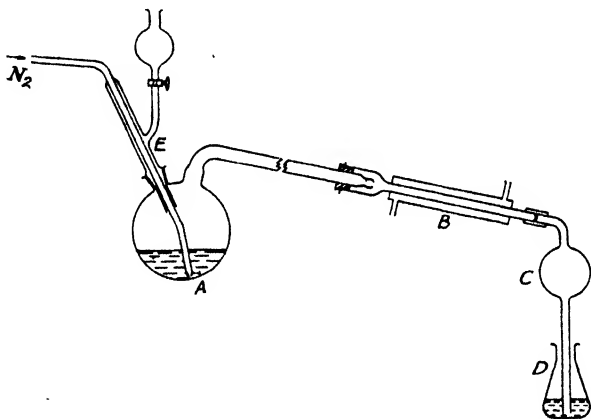


Fig. 3. Apparatus for recovery of isotopic ammonia

scribed above. When heating must be avoided, as in the case of glutamic acid, the distillation is carried out under reduced pressure, with the precautions described below.

Alanine—2.2 gm. of freshly distilled pyruvic acid were hydrogenated with 0.05 mole of ammonia containing 1.97 atom per cent N^{15} excess. 1.49 gm. of alanine (68 per cent of the theoretical amount) were obtained by precipitation with alcohol.

N (Kjeldahl) found 15.62 per cent; calculated 15.73 per cent.

It contained 1.87 atom per cent N^{15} excess.

Phenylalanine—1.64 gm. of phenylpyruvic acid prepared by condensation of benzaldehyde and acetylglycine according to Shemin and Herbst (12) were reduced in the presence of 0.02

mole of ammonia containing 1.97 atom per cent N^{15} excess. The product crystallized during hydrogenation. 1.38 gm. (84 per cent of theory) were obtained.

N (Kjeldahl) found 8.55 per cent; calculated 8.49 per cent.

It contained 1.89 atom per cent N^{15} excess.

Tyrosine—6.63 gm. of *p*-hydroxyphenylpyruvic acid prepared according to Dakin (13) were reduced in the presence of 0.074 mole of ammonia containing 1.97 atom per cent N^{15} excess. The tyrosine precipitated during hydrogenation. 5.51 gm. (82.8 per cent of theory) were obtained.

N (Kjeldahl) found 7.67 per cent; calculated 7.74 per cent.

It contained 2.04 atom per cent N^{15} excess.

Norleucine—A solution of 56 gm. of α -bromo-*n*-caproic acid in 400 cc. of water and 56 gm. of sodium carbonate was boiled for 30 minutes over a free flame. The mixture was acidified, extracted with ether, and the ether brought to a small volume. On the addition of ligroin, α -hydroxycaproic acid crystallized out in shiny plates. After one recrystallization the material melted at 59.5–60.5° (14). The yield was 25.6 gm. or 70 per cent of theory. The ethyl ester was prepared by refluxing with absolute ethyl alcohol and concentrated sulfuric acid.

23 gm. of hydroxy ester were dissolved in 200 cc. of glacial acetic acid and a solution of 10 gm. of chromic oxide in 50 cc. of 50 per cent aqueous acetic acid was slowly added with cooling and stirring. The mixture was allowed to stand overnight at room temperature, diluted with water, and extracted several times with ether. The ether extracts were washed with aqueous sodium carbonate, and dried with sodium sulfate; the ether was removed by distillation. The residual oil was distilled *in vacuo* at 4 mm. The fraction boiling at 55–65° amounted to 18 gm. or 79 per cent of theory.

The α -keto ester was saponified at room temperature with 1 equivalent of NaOH and the free acid isolated, after acidification, by extraction with ether. After removal of the ether the residual oil was distilled; b.p. 94–96° at 15 mm. Yield 5.9 gm. or 40 per cent of theory.

1.40 gm. of α -keto-*n*-caproic acid were reduced in the presence of 0.02 mole of ammonia. Norleucine precipitated during the hydrogenation. Yield 1.02 gm., 73 per cent of theory.

N (Kjeldahl) found 10.78 per cent; calculated 10.68 per cent. It contained 1.90 atom per cent N^{15} excess.

Aspartic Acid—2 gm. of oxalacetic acid prepared according to Wohl and Claussner (15) were reduced in the presence of 0.45 mole of ammonia. Yield 0.88 gm., 44 per cent of theory.

N (Kjeldahl) found 10.40 per cent; calculated 10.53 per cent. It contained 1.80 atom per cent N^{15} excess.

Glutamic Acid—7.30 gm. of α -ketoglutaric acid prepared according to Neuberg and Ringer (16) were reduced in the presence of 0.15 mole of ammonia. About 150 cc. of alcohol were added, the mixture was cooled to -60° , and the solution decanted from the catalyst into a Claisen flask connected with two traps containing dilute sulfuric acid. An excess of barium hydroxide was added to the mixture and the ammonia distilled into the traps *in vacuo* at $25-30^\circ$. The free amino acid was isolated from the barium salt in the usual manner. Yield 6.19 gm., 84.2 per cent of theory.

N (Kjeldahl) found 9.31 per cent; calculated 9.52 per cent.

It contained 1.95 atom per cent N^{15} excess.

Procedure B. Synthesis of Amino Acids with Potassium Phthalimide. Preparation of Potassium Phthalimide—Ammonia from 0.40 mole of isotopic ammonium salt in 200 cc. of water was slowly liberated by addition of potassium hydroxide in a Claisen flask as shown in Fig. 1 and distilled with a stream of nitrogen into a suspension of 68 gm. of phthalic acid (0.41 mole) in 200 cc. of water. The flask containing the suspension was also connected with a gas wash bottle containing H_2SO_4 to collect any ammonia not absorbed by the phthalic acid. Finally about 100 cc. of water were distilled over from the Claisen flask. By this time the ammonium hydrogen phthalate was all in solution. The solution was transferred to a 1 liter round bottom flask which had a neck 75 cm. long and 1.6 cm. wide. At a point 10 cm. from the upper end of the neck a side arm, 12 mm. internal diameter, is attached. This is bent at right angles 25 cm. from the flask and is then jacketed with cold water. The lower end of the side arm enters a suction flask, to the side arm of which is attached a bulb and a trap containing dilute sulfuric acid. After most of the water had been distilled over with a flame, the reaction mixture was heated in a metal bath and the temperature slowly raised to

200° until all the water was removed. The temperature was then slowly raised to 300°. When the vigorous reaction was complete, the flask was cooled, the neck was cut off, and the phthalimide removed with cold absolute alcohol. Yield 56.4 gm., or 96 per cent of theory.

The potassium salt was prepared as described by Salzberg and Supniewski (17). The yield was 92 per cent.

Recovery of Ammonia—The alcoholic mother liquors from the preparations of phthalimide and potassium phthalimide were combined with the contents of the acid traps. An excess of alkali was added and the ammonia distilled into sulfuric acid. 4 hours of boiling were necessary. The recovery of isotopic nitrogen was quantitative.

Glycine—18.6 gm. of the isotopic potassium phthalimide and 14 gm. of ethyl chloroacetate were heated for 2 hours at 150°. The reaction product was extracted with 150 cc. of absolute alcohol and treated with charcoal. After being concentrated and cooled, the solution yielded 21.2 gm. of ethyl phthalimidoacetate, m.p. 114°, or 91 per cent of the theory. The nitrogen of the mother liquor was quantitatively recovered as ammonia, by treatment with strong alkali.

15.2 gm. of the phthalimide ester were refluxed for $2\frac{1}{2}$ hours with a mixture of 68 cc. of concentrated hydrochloric acid, 68 cc. of glacial acetic acid, and 68 cc. of water. A fraction boiling below 108° was distilled off with the aid of a fractionating column and boiling was continued under a reflux for 12 hours. After cooling, the phthalic acid was filtered off and the filtrate brought to dryness *in vacuo*. The residue was extracted with a small amount of cold water. The solution was diluted, treated with silver carbonate, and filtered. The filtrate was treated with hydrogen sulfide, filtered, and brought to a very small volume. The glycine precipitated with alcohol weighed 5.01 gm., 98 per cent of theory.

Three samples of glycine were prepared by this method, containing 0.226, 1.09, and 6.70 atom per cent N^{15} excess respectively.

Preparation of Deuteroleucine (N^{15})—Commercial isocaproic acid contains appreciable amounts of the isomers *n*-caproic acid and ethylmethylpropionic acid which are difficult to remove and give rise to leucine contaminated with norleucine and isoleucine. The

pure compound was, therefore, prepared from isobutyl alcohol (b.p. 106.8–107.5°) by the malonic ester method (18).

Deuteroisocaproic Acid—12 cc. of 55 per cent D_2O and 4.5 gm. of $PtO_2 \cdot H_2O$ were placed in each of two 100 cc. round bottom flasks and the platinum oxide was reduced with deuterium gas. 20.3 gm. of isocaproic acid and 0.9 gm. of KOH were added, and the flasks sealed and shaken for 12 days at 125–130°. The flasks were opened, the contents acidified with phosphorus pentoxide, extracted with petroleum ether, and the residue distilled at atmospheric pressure. It boiled at 200.2°.

Ethyldeutero- α -Bromoisocaproate—76 gm. of deuteroisocaproic acid and 26.4 gm. of dry red phosphorus were treated with 408 gm. of bromine in the usual manner. The excess bromine was distilled off and the reaction mixture treated with absolute ethanol. The mixture was extracted with ether, and the solution washed with dilute aqueous $NaHCO_3$, dried with Na_2SO_4 , and the ether removed by distillation. The residual liquid was distilled *in vacuo*. It boiled at 83–84° (9 to 10 mm.); the yield was 103 gm.

Deuteroleucine (N^{15})—8 gm. of cupric oxide and 49.5 gm. of potassium phthalimide (0.27 mole) were ground together in a mortar and treated under a reflux with 66 gm. (0.30 mole) of ethyldeutero- α -bromoisocaproate in a metal bath at 200° for 3½ hours. The product was exhaustively extracted with hot ligroin. The ligroin and the unchanged bromo ester were removed by distillation.

The residual oil, consisting chiefly of ethyl- α -phthaliminoisocaproate was hydrolyzed, without further purification, by refluxing for 4 hours with a mixture of 130 cc. each of glacial acetic acid, concentrated hydrochloric acid, and water. The condenser was then replaced by a fractionating column and the homogeneous mixture was slowly distilled until the vapor temperature had risen to 105°. Refluxing was resumed for 10 hours longer. When cold, the phthalic acid was removed and the filtrate concentrated to a small volume *in vacuo*. A second crop of phthalic acid was filtered off and free leucine isolated from the filtrate after treatment with silver carbonate in the usual manner. The yield was 25.4 gm., or 72 per cent of theory.

N (Kjeldahl) found 10.57 per cent; calculated 10.68 per cent.

It contained 6.49 atom per cent N^{15} excess and 3.87 atom per cent deuterium.

The nitrogen from the mother liquors of the amino acid preparations was recovered quantitatively as ammonia by subjecting the residues to the Kjeldahl procedure.

SUMMARY

1. Methods are described for the preparation of amino acids containing nitrogen and hydrogen isotopes.

2. Two methods have been used for the synthesis of amino acids containing an excess of the nitrogen isotope N^{15} , starting with isotopic ammonia: Procedure A, the hydrogenation of α -keto acids in the presence of ammonia according to Knoop and Oesterlin, and Procedure B, the phthalimide procedure of Gabriel and Kroseberg. Both methods have been modified with the aim of obtaining a quantitative recovery of the isotope and avoiding a dilution with ordinary nitrogen.

3. The following isotopic amino acids were prepared: *dl*-alanine, *dl*-phenylalanine, *dl*-tyrosine, *dl*-norleucine, *dl*-glutamic acid, and *dl*-aspartic acid, all containing an excess of about 1.9 atom per cent N^{15} , as well as three glycine preparations with an excess of 0.226, 1.09, and 6.70 atom per cent N^{15} respectively.

4. *dl*-Leucine is described containing 6.49 atom per cent N^{15} excess as well as 3.87 atom per cent deuterium bound to carbon.

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STUDIES IN PROTEIN METABOLISM

IV. THE STABILITY OF NITROGEN IN ORGANIC COMPOUNDS*

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In work with deuterium as an indicator for the metabolism of organic compounds only substances could be employed in which the deuterium atoms were stably bound to the carbon chain of the molecule.

For the same reason nitrogen isotopes can be used for tracing amino acids in the organism only if the carbon-nitrogen linkage in amino groups is stable. If this nitrogen were exchangeable with other nitrogen, this would considerably restrict the biological application of the nitrogen isotope, as it might lead, by mere exchange, to a random distribution of the isotope in all amino acids of the animal.

The use of isotopes is the only direct method to investigate exchange reactions. The application of the oxygen isotope O^{18} has shown the oxygen of ketones to exchange readily with that of water though that of OH groups in organic compounds is stable (1). The iodine in methyl iodide exchanges with iodine ions in aqueous solutions (determined by the use of radioactive isotopes) (2) though the halogen in organic compounds of higher molecular weight is stable.

From the data of Urey and collaborators (3) and from our own experience it appears certain that gaseous nitrogen does not exchange with the nitrogen of ammonia. Isotopic ammonia has been heated in the presence of air, and nitrogen gas has been bubbled through boiling ammonia solutions (4), but no dilution

* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

of the isotope in the ammonia was observed. The same is true for the nitrogen in amino acids and in hippuric acid. Peptides presumably behave in the same way. We have not excluded air in the course of the organic preparations and have never observed a decrease of the isotope content in these substances.¹

For further investigation of the stability of nitrogen in organic compounds pairs of two different substances, only one of which contained an excess of isotope, were treated together in aqueous solution at various temperatures. At the end of the treatment that component was isolated which at the beginning had normal nitrogen. If an exchange reaction had occurred, it would have become apparent by the increased isotope content. We have, for methodological reasons, preferred to isolate the normal component of the system rather than the isotopic one. If the isotopic compound were analyzed, an accidental contamination with air of the nitrogen gas liberated would be interpreted as an exchange reaction.

The results are given in Table I.

The values obtained from all experiments with the exception of Experiments 5 and 9 are within the limit of error of the analytical procedure. They show that no appreciable exchange occurs in the system: (1) amino acid and ammonia (Experiments 1 to 3), (2) amino acid-amino acid (Experiment 4), (3) hippuric acid-amino acid (Experiments 6 and 7), (4) urea-amino acid (Experiment 10). The small increase of N^{15} in Experiment 5 is most probably due to a slight contamination (less than 1 per cent) of the isolated leucine by glycine and not to an exchange of their nitrogen atoms. It is more difficult to interpret the value 0.073 atom per cent in Experiment 9. It is a well recognized fact that aqueous solutions of urea, when heated, form equilibrium mixtures which contain ammonium and cyanate ions. In this reaction the presence of isotopic ammonia must inevitably result in the formation of isotopic urea. Under the conditions of our Experiment 9 (1 hour at 100°), the reaction is known to be slow. At 37° (Experiment 8) no exchange was observed; in Experiment 9 the urea

¹ The nitrogen atoms of NH_4^+ and NO_3^- do not exchange either. Urey and collaborators have concentrated the isotope in the ammonia of ammonium nitrate. The isotope concentration in the ammonia did not decrease even with long standing of the salt in solution.

acquired only 10 per cent of the isotope content which it should have had were the isotope distributed in a random manner over all the components of the system. On the other hand, part of the excess isotope in the urea may be attributed to the inclusion of a trace of ammonium salt in the bulky precipitate of dioxanthyl urea.

TABLE I
Exchange Reactions of Nitrogen

Experiment No.	System			Time hrs.	Temperature °C.	Excess N ¹⁵ atom per cent
	Normal component	Isotopic component				
			N ¹⁵ ex- cess			
	mg.	mg.	atom per cent			
1	Ammonium sulfate, 13.2	Glycine, 37.3	0.22	48	105	0.000
2	Glycine, 100	Ammonium chloride, 100	1.98	104	100	0.004
3	Arginine HCl, 220	Ammonium chloride, 500	1.98	104	100*	0.007†
4	Tyrosine, 248	Glycine, 159	0.22	48	105	0.003
5	Leucine, 203‡	" 200	1.21	104	100§	0.015
6	Hippuric acid, 283	" 214	0.22	48	50	0.003
7	" " 400	" 100	0.22	1	100	0.000
8	Urea, 400	Ammonium sulfate, 1000	0.22	48	37	0.000
9	" 100	" chloride, 100	1.98	1	100	0.073
10	" 400	Glycine, 1000	0.22	40	37	0.000

* The medium was adjusted to pH 4.7.

† This is the same abundance of N¹⁵ as was found in normal arginine (5).

‡ Isolated as copper salt.

§ The medium was 0.1 N KOH.

|| Isolated from the system with xanthidrol.

The experiments taken together clearly indicate that the nitrogen atoms in free amino acids, as well as in hippuric acid, are stable. The same is true for the guanido group of arginine. If an exchange occurs with urea, it must be a very slow reaction.

SUMMARY

The nitrogen atoms in amino acids, hippuric acid, and in the guanido group of arginine do not exchange with the nitrogen

of other compounds in aqueous solutions at 100°. The nitrogen of urea may be exchangeable, but at a relatively slow rate, even at 100°.

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STUDIES IN PROTEIN METABOLISM

V. THE UTILIZATION OF AMMONIA FOR AMINO ACID AND CREATINE FORMATION IN ANIMALS*

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There is no doubt that animals can synthesize certain amino acids, the ultimate source of the nitrogen being that of the food protein. This process probably occurs under normal conditions, as the average composition of food proteins is never the same as that of the body proteins into which they have to be converted. According to the generally accepted theory, amino acid synthesis in the animal follows the scheme by which ammonia reacts with α -keto acids to form imino acids which are reduced to amino acids. Knoop and Oesterlin (1) have followed this reaction in *in vitro* experiments.



According to this scheme amino acids could be formed either from ammonia administered directly to the animal or from that liberated by deamination of other amino acids.

A large amount of work has been carried out on the utilization of ammonia for protein synthesis in animals, but the results have not been conclusive. Partial replacement of protein nitrogen in the diet of laboratory animals or ruminants by ammonia or urea gave no proof of the utilization of these substances by tissue cells (2). Embden and Schmitz (3) by using a more direct method per-

* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

fused organs with α -keto acids and ammonia and obtained the corresponding amino acids. Neber (4) treated mixtures of pyruvic acid and ammonia with large quantities of tissue slices and observed a small increase of amino nitrogen above the control values. Although these experiments demonstrate amino acid synthesis by animal tissue, they offer no definite proof that the amino nitrogen formed was derived from the added ammonia. In all these experiments the perfused organs or the tissue slices contained amounts of protein nitrogen (or free amino nitrogen) far greater than those recovered in the newly formed amino acids. The new amino acid could have been formed by a direct shift of the amino nitrogen from an amino acid to the keto acid.

A reaction of this kind has recently been observed in *in vitro* experiments by Herbst (5). An amino acid treated with an α -keto acid gave rise to a new amino acid corresponding to the keto acid, a Schiff base being formulated as the intermediate step. Braunstein and Kritzmann (6) have recently advanced strong evidence that the reaction studied by Herbst actually occurs in organs: treatment of pyruvic acid with glutamic or aspartic acid and minced muscle gives rise to the formation of alanine and loss of glutamic acid. The authors suggest that these two aminodicarboxylic acids, or their corresponding keto acids, are intermediates in the formation of other amino acids. In contrast to the mechanism for amino acid formation, discussed above, the reactions of Herbst and of Braunstein do not involve ammonia.

The isotope of nitrogen can be applied to investigations of the rôle of ammonia in amino acid synthesis in animals. If isotopic ammonia is given, it will mix with the ammonia present in the animal organism, including that which is formed as an intermediate product. As the animal organ does not discriminate between the isotopes of the same element, both the ammonia administered and the ammonia formed in the cell must be treated alike. If ammonia is used in the synthesis, the newly formed amino acid should contain an increased amount of isotope. As administered ammonia is not directly excreted in the urine, but first converted into urea, it is involved in metabolic reactions. As will be shown later, it is available also for other syntheses.

We have carried out two exploratory experiments by feeding isotopic ammonia to animals under conditions in which amino acid

synthesis was to be expected. An adult rat was kept on a stock diet, the protein of which was casein. This does not contain appreciable amounts of glycine. About 300 mg. of benzoic acid were added to the diet for 10 days; this resulted in a continuous excretion of glycine (830 mg.) in the form of hippuric acid, for which an equivalent amount of glycine must have been synthesized. The diet also contained 1.2 per cent nitrogen (with 1.21 atom per cent N^{15} excess) in the form of ammonium citrate. The animal kept its weight and consumed the same amount of food as the controls on the same diet without benzoic acid and ammonium citrate.

The nitrogen of the urinary hippuric acid contained a small but definite increase of the nitrogen isotope (0.025 to 0.045 atom per cent), which indicates that at least a small amount of the dietary ammonia was utilized for the glycine synthesis. As the dietary ammonia contained 1.21 atom per cent N^{15} excess, about 3 to 4 per cent of the urinary glycine was formed by use of nitrogen of the administered ammonia. The creatine isolated from the carcass also contained a small but significant increase of the isotope.

A similar result was obtained in the second experimental series: Two immature rats of 60 gm. each were kept for 5 days on a low protein diet (3 per cent as yeast protein) which contained 2.3 per cent nitrogen as ammonium citrate (1.21 atom per cent N^{15} excess). The animals lost weight as was expected. These unphysiological conditions were chosen in the expectation that the rats in the need of protein might synthesize at least a moderate amount of amino acids by utilizing the isotopic ammonia. This was the case. The protein nitrogen of the carcass contained a slight excess of N^{15} above normal.

In order to locate the isotopic nitrogen in the body constituents, samples of the following seven different amino acids, in addition to creatine, were isolated in pure state: glycine, glutamic acid, aspartic acid, proline, histidine, arginine, and lysine. All these substances, with the exception of lysine, contained a small but definite excess of N^{15} . The isotope concentration in the lysine was normal (Table II).

The highest values were found in glutamic acid and in aspartic acid. It is interesting to note that the dicarboxylic acids, which according to the work of Braunstein and Kritzmann (6) seem to

play an especially active rôle in amino acid metabolism since they are intermediates in the formation of other amino acids, demonstrate this activity also by their high uptake of isotopic nitrogen. The findings on lysine and on the dicarboxylic acids are similar to those of the experiments in which animals were given heavy water (7). Glutamic and aspartic acids isolated from these animals had the highest deuterium content, while lysine was free of the isotope.

The arginine from the animals given isotopic ammonia also contained N^{15} . In order to locate the isotope in the molecule of this amino acid, the arginine isolated was hydrolyzed with strong alkali into ornithine and ammonia (8). The isotope was found in the ammonia, while the ornithine contained normal nitrogen. The isotope was thus present in the $HN=CNH_2$ part of the

guanido group of the arginine. It is the same part which is liberated as urea by arginase,¹ and which according to the theory of Krebs and Henseleit (9) is potential urea and is involved in normal urea formation. As ammonia given to animals is always converted into urea, our finding on arginine is additional support for this theory.

The high isotope content of the amide nitrogen of the protein is noteworthy. More than 10 per cent of this amide nitrogen was derived from the administered ammonia. According to the well accepted theory, the amide nitrogen in proteins is linked to the free carboxyl group of the combined dicarboxylic acids (as glutamine and asparagine). The result is of interest in connection with the finding of Leuthardt (10) that glutamine and asparagine may form urea independently of the ornithine cycle. The relation of urea formation to arginine and amide nitrogen will be discussed in a subsequent publication.

The experiments leave no doubt as to the ability of animals to utilize at least a small amount of ammonia for amino acid and creatine formation. However, the unphysiological conditions employed in both series make it uncertain that we are dealing with a process which takes place under normal conditions.

¹ We have not employed arginase for the splitting as the normal nitrogen in the enzyme solution would have diluted the isotope in the amino acid.

EXPERIMENTAL

Feeding Benzoic Acid and Isotopic Ammonia to an Adult Rat—A male rat of 198 gm. was kept in a metabolism cage on the following diet: casein 16, corn-starch 47, lard 25, yeast 5, salt mixture (Osborne and Mendel (11)) 4, and sodium benzoate 3 per cent. After 2 days on this diet the animal received for another 6 days an addition of isotopic ammonia (1.21 atom per cent N^{15} excess) as the citrate. The diet thus contained 3.5 per cent total nitrogen, of which 0.6 per cent was that of the isotopic ammonia. As the average daily food intake was 12.1 gm., the animal consumed 72 mg. of ammonia nitrogen daily. Its weight remained constant (197 gm. at the end of the experiment). The urine was collected

TABLE I
Isotope Content of Compounds from Rat Given Benzoic Acid and Ammonia N^{15} (1.21 Atom Per Cent Excess)

The isotope concentration in the hippuric acid isolated before the feeding of isotopic NH_3 was 0.002 atom per cent N^{15} excess.

Compound	Feeding period	N^{15} concentration
	days	atom per cent excess
Hippuric acid.....	5	0.040
“ “	6	0.038
“ “	8	0.043
Body creatine.....	9	0.025

under toluene and pure hippuric acid was isolated daily in the usual manner. From the urines of the 8 day period a total of 1.89 gm. of hippuric acid, corresponding to 0.83 gm. of glycine, was isolated. For the isotope determination the samples obtained on the 5th, 6th, and 8th days were analyzed separately. The creatine of the carcass was isolated as described later. The results are given in Table I.

Feeding a Protein-Low Diet and Isotopic Ammonia to Immature Rats—Two male rats of 72 gm. each were kept in a metabolism cage on a diet of the following composition: corn-starch 63.75, lard 28.35, yeast 5.65, salt mixture (11) 2.25 per cent. To the diet was added 2.25 per cent of nitrogen (1.21 per cent N^{15} excess) in the form of ammonium citrate. A sample of the total nitrogen

of the diet was analyzed for its isotopic composition. It contained 0.83 atom per cent N^{15} excess; *i.e.*, about 70 per cent of the dietary nitrogen was present as ammonia (1.21 atom per cent N^{15} excess). The animals were kept on this diet for 5 days. They had consumed at the end of the experiment a total of 40 gm. of the diet, corresponding to an average of 180 mg. of ammonia nitrogen (1.4 gm. of N per kilo of body weight) per day.

The average weight of the animals had dropped from 72 gm. to 60.6 gm.; *i.e.*, each animal lost about 2.2 gm. per day. The rats were killed; the intestinal tract was removed, washed with water, and added to the rest of the carcasses. The combined material (120 gm.) after being cut into small pieces was extracted three times with boiling alcohol for 8 hours each. The combined alcohol extracts were used for the isolation of creatine as described later.

Isolation of Amino Acids—The extracted carcasses were dried (weight 29 gm.) and boiled for 20 hours with 20 per cent hydrochloric acid. The solution was made neutral to phenolphthalein with barium hydroxide and the precipitate filtered off, whereby most of the calcium and phosphates of the skeleton were removed. From an aliquot of the solution free ammonia was distilled off *in vacuo* for the isotope analysis of the "amide nitrogen." The remaining solution was freed of barium, and samples of the following amino acids were isolated by procedures previously described (7): glycine, glutamic acid, aspartic acid, proline, histidine, lysine, and arginine. The samples were recrystallized until pure (Kjeldahl analysis).

Splitting of Isotopic Arginine—24 mg. of the arginine monohydrochloride were boiled for 6 hours with 50 per cent KOH. A slow stream of nitrogen gas carried the liberated ammonia into a receiver containing dilute H_2SO_4 . Isotope analyses were carried out with the ammonia solution as well as with the alkaline solution containing the ornithine.

Isolation of Creatine As Potassium Creatinine Picrate from the Carcasses—The alcoholic extract of the carcasses was evaporated to a small volume *in vacuo*, and the aqueous residue made acid (approximately 1 N) with hydrochloric acid and extracted with ether to remove fats. It was boiled for 4 hours to convert creatine into creatinine, brought to dryness *in vacuo*, and the residue dis-

solved in 300 cc. of water. It was cleared by adding a solution of 1 gm. of lead acetate followed by NaOH until just alkaline to phenolphthalein; lead was removed from the filtrate by hydrogen sulfide. The solution was made faintly acid to Congo red with hydrochloric acid, and treated with 0.5 gm. of picric acid and 0.5 gm. of potassium picrate, which were dissolved by warming. The crystals formed after 2 days standing in the ice box were recrystallized from 300 cc. of a solution containing 0.12 per cent of picric acid and 0.12 per cent of potassium picrate. In this solution at ice temperature potassium creatinine picrate is practically insoluble

TABLE II

Compounds from Immature Rats Given Low Protein Diet and Isotopic Ammonia (1.21 Atom Per Cent N¹⁵ Excess)

	N ¹⁵ excess
	atom per cent
Amide N.....	0.114
Glycine.....	0.050
Glutamic acid.....	0.085
Aspartic ".....	0.067
Proline.....	0.037
Histidine.....	0.012
Lysine.....	0.003
Arginine.....	0.033
"Urea" from arginine.....	0.069
Ornithine from arginine.....	0.004
Creatine.....	0.036

and recrystallization can be repeated many times without appreciable loss. The final product, 0.703 gm. (from the second series), was assayed colorimetrically for its creatinine content; found 18.5 per cent, calculated 18.6 per cent. N (Kjeldahl after reduction with Sn + HCl) found 20.8, 20.6; calculated 20.7. For the isotope analysis of the creatinine a sample of the potassium creatinine picrate was freed of picric acid by suspending in dilute H₂SO₄ and extracting with ether. The analysis was carried out with the aqueous solution.

All values obtained in the isotope analyses of the compounds from the animals are given in Table II.

DISCUSSION

All the isotope values listed in Tables I and II, with the exception of that of lysine and ornithine in Table II, are above normal. The increase of N^{15} is far above the limit of error discussed in Papers I and II of this series.

The isotope was given to the animals in the form of ammonia and could not have entered the organic compounds in the animals by mere physical exchange, as the nitrogen in the compounds is stably bound. This is further shown by the negative findings on lysine (and on ornithine). If the presence of isotope in the amino acids were due to physical exchange, it should be found in all amino acids, including lysine, as the carbon-nitrogen linkage at the α -carbon atoms is the same. The isotopic nitrogen must have entered by a chemical reaction, either by a new formation of amino acids from substances with different carbon chains or by successive deamination and amination of the same carbon skeleton (see discussion in (7)).

The results of both experiments offer definite proof for the ability of rats to utilize at least a small amount of dietary ammonia. They suggest that ammonia liberated from amino acids may be utilized for the formation of other amino acids. They do not, however, exclude other mechanisms, such as the direct transfer of nitrogen from an amino acid to a keto acid as discussed before.

Both experiments were unphysiological. Hippuric acid formation is a normal process in herbivorous animals, but the amount of benzoic acid detoxified in our first experiment is much higher than is ever observed normally. This fact, together with the peculiar conditions under which the rats were kept in the second experiment, leaves it uncertain whether the observed slight utilization of ammonia plays an appreciable rôle under normal conditions.

SUMMARY

1. Two experiments were carried out on the utilization of ammonia for amino acid synthesis in rats by feeding ammonia N^{15} .
2. An adult rat was given a diet containing 16 per cent casein, together with benzoic acid and isotopic ammonia in the form of ammonium citrate. While the diet was practically devoid of glycine the animal excreted in the 8 day period more than 830 mg. of glycine in hippuric acid. At least part of this glycine must have

been synthesized. The nitrogen of the hippuric acid showed a small but definite increase of its isotope content, demonstrating the utilization of a small amount of dietary ammonia for glycine formation.

3. Two immature rats were fed a low protein diet to which a large amount of isotopic ammonia was added as the citrate. Creatine, glycine, glutamic acid, aspartic acid, proline, histidine, lysine, and arginine were isolated from the bodies of the animals. All, with the exception of lysine, contained an excess of isotope, the highest concentration being found in glutamic and aspartic acids. The animals had thus utilized a small amount of the dietary ammonia.

4. The ammonia liberated during protein hydrolysis ("amide nitrogen") had an isotope concentration much higher than that of any amino acid.

5. The arginine from the animals was hydrolyzed into ammonia and ornithine. The isotope was found in the ammonia, while the ornithine moiety had normal nitrogen. The isotope was thus present in the guanido group of the arginine.

6. The isotope content in the creatine of the tissues from both experiments was also above normal.

7. The experiments offer proof for the ability of rats to utilize at least a small amount of ammonia for amino acid and creatine formation.

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STUDIES IN PROTEIN METABOLISM

VI. HIPPURIC ACID FORMATION STUDIED WITH THE AID OF THE NITROGEN ISOTOPE*

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Benzoic acid when given to mammals is detoxified by being coupled with glycine to form hippuric acid. This reaction goes on even when no glycine is present in the food. Animals can supply more glycine for this reaction than is present in the body—definite proof of the ability of animals to synthesize this amino acid. Dietary glycine, however, increases the tolerance for benzoic acid (1).

The present experiment is concerned with the question as to whether dietary glycine is used directly for hippuric acid formation or whether tissue glycine is preferably employed. The results show that even when an excess of glycine is supplied, animals utilize only a very limited amount of the exogenous amino acid for hippuric acid formation, the bulk of the glycine being supplied by the animal.

The use of the nitrogen isotope has made it possible to distinguish between food and body glycine in an animal experiment. If glycine N^{15} is administered, the course of this amino acid can be followed by its isotope content. It has been shown previously (2) that the nitrogen in glycine is stably bound and does not exchange with the nitrogen of other nitrogenous compounds unless a chemical reaction is involved. If isotopic glycine is given and normal glycine isolated from the excreta, this may be taken as an indication that the excreted material was not derived directly from the food. If the excreted material contains some of the

* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

labeled nitrogen, the dilution factor of the isotope (ratio of the isotope in dietary to the isotope in urinary glycine) is a measure of how much of the urinary glycine was derived from the food and how much from the tissues.

Adult rats previously kept on the usual stock diet were given 300 mg. of benzoic acid either by stomach tube or by injection. Together with the benzoic acid an excess of glycine N^{15} (2 moles of glycine per mole of benzoic acid) was administered. The urinary hippuric acid in all cases contained an excess of N^{15} , indicating that administered glycine was utilized (Table I). The isotope concentration in all samples of the hippuric acid was, however, only about one-third of that in the administered glycine.

TABLE I
Hippuric Acid Formation

Experiment No.	No. of animals	N^{15} content in administered glycine	N^{15} content in hippuric acid	Dilution factor of labeled N in hippuric acid
		<i>atom per cent excess</i>	<i>atom per cent excess</i>	
1	2	0.21*	0.08	2.6
2	2	0.21†	0.07	3.0
3	5	1.06†	0.48	2.2

* Solution given by stomach tube.

† Solution given by injection.

Despite the excess of administered glycine, about two-thirds of the urinary glycine must thus have had another origin. The results do not indicate whether the glycine was taken from the proteins or was directly synthesized for the detoxification process. They do, however, point out that the organs supply the greater part of this amino acid, even when an excess is administered. There was no significant difference in the results whether the glycine and benzoic acid were injected (Experiments 2 and 3) or given by stomach tube (Experiment 1).

EXPERIMENTAL

The isotopic glycine was prepared as previously (3). All experiments were carried out with male rats weighing about 200 gm. which had been fasted for 12 hours previous to the experiment.

They were kept in metabolism cages and the urine was collected during 24 hours following the administration of the isotopic compounds. The urine of the animals in each experiment was pooled and pure hippuric acid isolated in the usual manner.

An amount of benzoic acid corresponding to 300 mg. per rat was given by stomach tube or by injection as sodium benzoate. The solution (about 3 cc.) contained 2 moles of isotopic glycine per mole of benzoic acid.

The data obtained are given in Table I.

SUMMARY

1. Biological hippuric acid formation in rats was studied with glycine preparations containing labeled nitrogen.
2. Glycine given with benzoic acid either by mouth or by injection is utilized for the hippuric acid formation.
3. However, only part of the administered glycine, even if given in excess, is used for this reaction, the major part being supplied by the animal tissues.

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STUDIES IN PROTEIN METABOLISM

VII. THE METABOLISM OF TYROSINE*

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In this paper we report the first experiment in which one amino acid of a normal diet, tyrosine, has been labeled by the nitrogen isotope. The experiment was carried out with a full grown rat kept for 10 days on the ordinary stock diet, the protein of which consisted mainly of casein. The tyrosine of this diet was labeled by adding to it an amount of synthetic, isotopic *dl*-tyrosine corresponding to twice that already present in the food. The isotope content in the added tyrosine was so high (2.04 atom per cent N^{15} excess) that its nitrogen could be diluted with several hundred times the amount of ordinary nitrogen and still be detectable by the analytical methods. We feel confident that we have carried out an experiment under physiological conditions, as the amino acid composition of natural diets differs widely and animals are accustomed to considerable variations in the amounts of the component amino acids.

The original aim of this exploratory experiment was merely to find out whether in nitrogen equilibrium the nitrogen in the urine is derived from the food proteins directly or whether dietary nitrogen is deposited, with liberation of an equivalent amount of tissue nitrogen for excretion.

In our experiment only part (50 to 60 per cent) of the labeled nitrogen given in the *dl*-tyrosine was recovered from the urine, while almost all of the rest was found in the tissues. The amount of labeled nitrogen deposited in the organs was so large that the

* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

original scope of the experiment was widened, and an attempt was made to trace the labeled nitrogen in the body constituents of the animal.¹

The liver, the blood, and the rest of the carcass were worked up independently in such a way as to separate the non-protein nitrogen from the protein nitrogen. Twenty-one different fractions or pure compounds were isolated and their isotope content analyzed. Almost all of the labeled nitrogen retained in the animal was found in the proteins and only small quantities in the non-protein nitrogen fractions. The concentration of labeled nitrogen in the liver was much higher than in the remaining carcass, a finding in agreement with the well established theory that the liver plays an important rôle in protein metabolism. As no other organ, such as the kidney or the intestinal tract, was investigated separately, it cannot be decided whether the liver differs in this respect from other internal organs. The blood corpuscles contained almost no labeled nitrogen, but the isotope concentration in the plasma fell between that of the total carcass and that of the liver.

The samples of pure tyrosine isolated from the liver protein and from the carcass protein contained a high concentration of labeled nitrogen; *i. e.*, some dietary tyrosine was directly deposited in the tissue proteins. The amount of the isotope in the total tyrosine of these proteins, however, accounted for only 25 to 30 per cent of the total labeled nitrogen in the proteins. The major part was thus present in other protein constituents.

The isotope, apart from tyrosine, could be located in four different places: (1) in the α -amino group of other amino acids; namely, in the dicarboxylic acid fraction (glutamic and aspartic acids) which was secured from the proteins both of the liver and of the carcass; (2) in histidine isolated from the liver proteins; (3) in the arginine obtained from the liver proteins. The labeled nitrogen could be located in that part of the guanido group which represents "potential urea" (split off as urea by arginase). The

¹ At the time when the feeding was started the amount of isotope available limited the experiment to one animal. The administration of an isotopic compound to only one rat can give direct evidence of the ability of the animal to convert one substance into another. Some of the reactions discussed below are certainly typical for the normal metabolism of tyrosine.

ornithine moiety of the arginine contained no labeled nitrogen; (4) in the "amide nitrogen" (liberated as ammonia during proteolysis) in the proteins from liver and from carcass.

In all these substances the concentration of labeled nitrogen was much lower than in the tyrosine, but far above the limit of error of the analytical method.

The only amino acid isolated which contained no labeled nitrogen was lysine.

The amount of labeled nitrogen found in the few fractions investigated (tyrosine, dicarboxylic acids, arginine, histidine, and "amide nitrogen") still accounted for only a portion of the total isotope in the proteins. Other amino acids which were not isolated must also have contained labeled nitrogen.

The results indicate that in our rat the nitrogen of at least one of the dietary amino acids, tyrosine, was only partly excreted in the urine, while almost half of it was retained in the body proteins. Of this deposited nitrogen, only a fraction was found attached to the original carbon chain, namely to tyrosine, while the bulk was distributed over other nitrogenous groups of the proteins.

EXPERIMENTAL

A 15 month-old male rat weighing 345 gm. was kept for 6 days on the following diet: casein 15, corn-starch 68, yeast 5, salt mixture (Osborne and Mendel (1)) 4, cod liver oil 2, and Wesson oil 6 per cent. The total protein of the diet contained about 4.5 per cent tyrosine. After 6 days an amount of isotopic *dl*-tyrosine (2.04 atom per cent N^{15} excess) corresponding to double that in the dietary protein was added to the diet. It was dissolved in a small volume of water with an equivalent amount of Na_2CO_3 . The animal received this addition for 10 days while the urine was collected. At the end of this period the animal had consumed a total of 1.867 gm. of the added tyrosine corresponding to 144.4 mg. of N. The animal did not spill food and its weight remained practically constant. (The weight at the end of the period was 350 gm.)

The animal was anesthetized with ether, and 10 cc. of blood were collected by heart puncture. It was killed and the total digestive tract removed and discarded. The liver was worked up separately from the remaining carcass. All samples were treated

in such a way as to separate the non-protein nitrogen from the protein.

Fractionation of Blood—10 cc. of the oxalated blood were centrifuged and the plasma was separated from the corpuscles. 5 cc. of plasma were treated with 50 cc. of 6 per cent trichloroacetic acid, and the precipitate filtered and washed with 6 per cent trichloroacetic acid. The precipitate (protein fraction) containing 47.5 mg. of N and the filtrate (non-protein fraction) containing 1.8 mg. of N were used for isotope analysis. The corpuscles were treated in an analogous manner. The non-protein nitrogen from the corpuscles was lost.

Fractionation of Liver—The liver (9.5 gm.) was frozen with solid carbon dioxide, pulverized in a steel mortar, and extracted with 6 per cent trichloroacetic acid. The filtrate (containing 16.5 mg. of N) was used for the analysis of the non-protein nitrogen and the precipitate (containing 224 mg. of N) for the isolation of amino acids.

Amino Acids from Liver Protein. Tyrosine—The precipitate was hydrolyzed with 15 cc. of 20 per cent hydrochloric acid and an aliquot taken for the isotope analysis of protein nitrogen. The hydrochloric acid was removed by distillation and by treatment with silver carbonate. The hydrolysate was finally concentrated to about 5 cc. The tyrosine which crystallized during 2 days in the ice box was collected and recrystallized from hot water. 30 mg. of pure tyrosine were obtained.

N (Kjeldahl) found 7.37 per cent; calculated 7.64 per cent.

Isolation and Splitting of Arginine—The mother liquor was brought to a volume of 30 cc. and arginine was precipitated with flavianic acid and the flavianate recrystallized. The 270 mg. of substance were decomposed with H_2SO_4 and butyl alcohol. The aqueous layer containing the arginine was treated with charcoal. An aliquot of the aqueous solution was used for the isotope determination in the arginine and the rest was hydrolyzed into ammonia and ornithine as previously described (2).

"Amide Nitrogen"—After removal of excess flavianic acid with butyl alcohol and charcoal, an aliquot of the mother liquor of the arginine precipitation was made alkaline with $\text{Ba}(\text{OH})_2$, and the ammonia was distilled *in vacuo* into a receiver containing H_2SO_4 . The total amount of amide nitrogen of the liver protein was 10.7 mg.

Dicarboxylic Acids—The residual alkaline solution (30 cc.) was added to 120 cc. of ethanol. The precipitate formed overnight was filtered off and dissolved in 25 cc. of water. The solution was cleared with charcoal and again treated with 4 volumes of alcohol. The barium salts of the dicarboxylic acids were used for isotope analysis.

Histidine—Barium was removed from the mother liquor of the barium precipitation and the basic amino acids were precipitated with phosphotungstic acid. The washed phosphotungstates were decomposed by extracting the acidified solution with a mixture of amyl alcohol, ethyl alcohol, and ether (3). From the aqueous layer histidine was precipitated with HgCl_2 at pH 6.5 in ethanol (4). The washed precipitate was decomposed with H_2S and the precipitation was repeated. The HgCl_2 complex, containing 3.4 mg. of N, was used for the isotope analysis of histidine.

Lysine—After removal of mercury the mother liquor from the histidine precipitation was evaporated to dryness and the residue extracted with ethanol. On concentration to about 0.5 cc. lysine monohydrochloride was precipitated by addition of pyridine and alcohol. It was twice recrystallized by dissolving in water followed by precipitation with ethanol. The yield was 14 mg.

N (Kjeldahl) found 14.5 per cent; calculated 15.1 per cent.

Fractionation of Remaining Carcass—The carcass (from which blood, liver, and intestinal tract had been removed) was passed twice through a meat grinder and extracted with 1750 cc. of 6 per cent trichloroacetic acid at room temperature. The filtrate contained 947 mg. of N (non-protein nitrogen). The residue containing 8.94 gm. of N was hydrolyzed and 250 mg. of pure tyrosine were obtained by crystallization followed by two recrystallizations.

N (Kjeldahl) found 7.64 per cent; calculated 7.74 per cent.

Amide nitrogen (383 mg.) and the barium salts of the dicarboxylic acids were obtained as described for the liver.

Fractionation of Urine—The urines of the first 9 days containing 1.950 gm. of total N were pooled and a sample was used for isotope analysis. The urine of the last 24 hours was collected separately. Ammonia was absorbed on permutit and recovered from it by addition of strong alkali and distillation into acid. The filtrate from the permutit was treated with urease and the ammonia liberated was carried into acid by a stream of air. The urine

contained 167.5 mg. of total N, 140 mg. of urea N, and 3.0 mg. of ammonia N.

The isotope analyses of all the samples obtained from the animal are given in Table I.

Quantitative Determination of Tyrosine in Liver and Carcass of Rat—For the calculation of the amount of dietary tyrosine re-

TABLE I
*N¹⁵ Concentration in Nitrogen of Substances Isolated from Rat Given
dl-Tyrosine N¹⁵*

	Substance	N ¹⁵
		atom per cent excess
Liver	Total protein	0.039
	Tyrosine	0.316
	Arginine	0.030
	NH ₂ from arginine	0.058
	Glutamic + aspartic acid	0.056
	Lysine	0.003
	Histidine	0.015
	Amide N	0.044
Carcass	Non-protein N	0.038
	Total protein	0.012
	Tyrosine	0.100
	Glutamic + aspartic acid	0.010
	Amide N	0.012
Blood	Non-protein N	0.015
	Protein from plasma	0.029
	Non-protein N from plasma	0.066
	Protein from corpuscles	0.009
Urine	Total N first 9 days	0.077
	“ “ last 24 hrs.	0.067
	Urea last 24 hrs.	0.067
	NH ₂ “ 24 “	0.197

tained by the animal, the amount of total tyrosine in our rat must be known. We have determined by the method of Folin and Marenzi (5) in another rat the tyrosine content of the liver and of the carcass from which the liver and intestinal tract had been removed. According to these analyses the liver of our animal should have contained 77 mg. and the remaining carcass 2770 mg. of tyrosine.

DISCUSSION

Amount of Dietary Tyrosine Incorporated in Proteins—The incorporation of dietary tyrosine in the tissues of a normal animal recalls the results of experiments in which labeled fatty acids were given to normal mice (6). Even when the animals kept their body weight constant, a large fraction of the dietary fatty acids was deposited, with liberation of an equivalent amount of tissue fatty acids for combustion.

Almost all of the labeled tyrosine in our animal was in the proteins. The total amount of non-protein nitrogen and its isotope content were not large enough to account for a significant quantity of tyrosine.

From the isotope content of the tyrosine isolated and that of the diet, one can calculate the minimum amount of the dietary amino acid deposited in the proteins. The material consumed was a mixture of 0.93 gm. of ordinary tyrosine in the food proteins and 1.86 gm. of added isotopic material with 2.04 atom per cent N^{15} excess. A total of 2.79 gm. of tyrosine with 1.36 atom per cent N^{15} excess was thus consumed. The liver protein of the animal contained 77 mg. of tyrosine with 0.32 atom per cent N^{15} excess, and the remaining carcass 2770 mg. of tyrosine with 0.10 atom per cent. From the dietary tyrosine there were thus derived 24 per cent or 18 mg. of the liver tyrosine and 7 per cent or 204 mg. of the carcass tyrosine. A total of 222 mg. or 8 per cent of the consumed tyrosine was recovered from the tissue proteins.

In the calculations no account was taken of the fact that the isotopic tyrosine added to the diet was racemic. The metabolism of amino acids of unnatural steric configuration is known to be different from that of the natural isomerides; they are not deposited in the protein as such. From work on indispensable amino acids, it is known that some of the unnatural acids may substitute for the natural ones in the diet; *i.e.*, they may be converted. On the basis of the current conception of such conversions (deamination and amination of the corresponding keto acid) as well as of experiments to be published later, it is improbable that the nitrogen originally present would have remained attached to the carbon chain during this reaction. In our experiment, if the conversion had occurred, the labeled nitrogen of the unnatural *d*(+)-tyrosine would probably have been replaced by normal

nitrogen, thus diluting the isotope concentration of the deposited tyrosine. If the animal had received the natural *l*(-)-tyrosine only, the amount of labeled tyrosine deposited would probably have been considerably higher than 8 per cent.

Isotope Content in Protein Molecule Apart from Tyrosine. Liver Protein—This protein contained 77 mg. of total tyrosine, corresponding to 5.9 mg. of N. The isotope in this nitrogen was 0.316 per cent or 0.018 mg. of N^{15} excess. The total protein nitrogen of the liver, 224 mg., contained 0.039 per cent or 0.087 mg. of N^{15} excess. Thus 0.069 mg. of N^{15} excess (0.087 - 0.018 mg.) must have been present apart from tyrosine; *i.e.*, only about 25 per cent of all the labeled nitrogen in the protein was present as tyrosine.

Carcass Protein—This protein contained 2770 mg. of total tyrosine, corresponding to 213 mg. of N. The isotope content in this nitrogen was 0.10 per cent, or 0.21 mg. of N^{15} excess. The total protein contained 8900 mg. of nitrogen with 0.012 per cent or 1.06 mg. of N^{15} excess. Thus 0.85 mg. of N^{15} excess must have been present apart from tyrosine; *i.e.*, only about 20 per cent of all the labeled nitrogen in this protein was present as tyrosine.

The last value obtained for the carcass protein includes a somewhat larger error than that for the liver, as the value for the isotope concentration in the carcass protein is rather low. But even a considerable error could not alter the conclusion that also in this protein only a fraction of the labeled nitrogen was present as tyrosine.

Dicarboxylic Acids—These were the only monoamino acids investigated. The labeled nitrogen must have been derived from nitrogen of the dietary tyrosine. The result does not indicate whether tyrosine was directly converted into the dicarboxylic acids, *i.e.* whether the carbon was also utilized, or whether the nitrogen only was involved, but the utilization of the carbon chain for the formation of dicarboxylic acids is highly improbable. It is more likely that the nitrogen only was used, for example for the amination of the corresponding keto acid. Whatever the mechanism may be, the result must be taken as proof that our animal, which was fed a stock diet and was kept in nitrogen equilibrium, had formed new amino acid molecules, even such as were abundant in the diet (casein contains 4.1 per cent of aspartic acid and 22 per cent of glutamic acid). Any reaction which led to the introduc-

tion of isotopic nitrogen into these monoamino acids must have been accompanied by an opening of the peptide linkage by which the nitrogen atom was bound.

From the isotope concentration in the dietary tyrosine and that of the dicarboxylic acids the minimum amount of dicarboxylic acids in the liver which were newly formed by using tyrosine nitrogen can be calculated. As the tyrosine contained 1.36 and the dicarboxylic acids 0.056 atom per cent N^{15} excess, the fraction of the newly formed compounds was 4.3 per cent.

Tyrosine is only one of the amino acids of casein, and the tyrosine nitrogen represented only 6 per cent of the total protein nitrogen in our diet. It is possible—though improbable—that tyrosine was the only amino acid the nitrogen of which was used for these synthetic reactions. The nitrogen of the other amino acids was not labeled, and a transfer of it to the dicarboxylic acids would not have been detected in our study. If the tyrosine nitrogen utilized for the synthesis represents only part, the percentage of newly formed dicarboxylic acids must have been correspondingly higher.

Histidine—We have not yet located the position of the labeled nitrogen in the 3 nitrogen atoms of this amino acid. As histidine is an indispensable food constituent, it is likely that no chemical reaction had occurred whereby ring nitrogen was replaced by isotopic nitrogen. The labeled nitrogen was probably attached almost exclusively to the α -carbon atom and may have been introduced by successive deamination and amination in a way such as has been suggested (7) for the introduction of stable deuterium into histidine in the living animal.

Arginine—The arginine isolated from the liver protein contained 0.030 atom per cent N^{15} excess. The ammonia obtained by alkaline hydrolysis contained double this concentration (0.058 atom per cent). As 2 nitrogen atoms are split off from a total of 4 in this reaction, all labeled nitrogen must have been present in that part of the molecule which was recovered as ammonia, and represents "potential urea." The ornithine moiety, which in this experiment was not analyzed, must have contained only normal nitrogen. This result is analogous to that in the previous experiment (2), in which animals were given labeled ammonia instead of tyrosine.

In the present experiment the arginine was isolated from the

liver, while in the earlier one it was obtained from the total carcass. The ammonia (or "potential urea") from the liver arginine had a concentration of labeled nitrogen (0.058 atom per cent N^{15} excess) almost as high as that of the urinary urea (0.067 atom per cent N^{15} excess). This may be taken as even a better support of the theory of Krebs and Henseleit (8) than the results of the previous experiment, as urea formation is supposed to occur only in the liver.²

Amide Nitrogen—The isotope concentration of this fraction obtained from the liver was only slightly lower than that of the "potential urea" in arginine. Leuthardt (9) has given evidence that glutamine and asparagine form urea independently from the ornithine cycle. Our amide nitrogen was obtained from the protein; *i.e.*, the amide groups were attached to dicarboxylic acids in protein linkage.³

Lysine—The lysine isolated from the liver was the only substance which contained no labeled nitrogen. This finding is in agreement with the results of all earlier experiments, in which isotopes have been applied to the study of amino acid metabolism. Of all amino acids investigated lysine was the only one which did not take up deuterium from heavy water in the body fluids (7), and in one of the preceding papers (2) it was shown to be the only substance which does not take up N^{15} from isotopic ammonia given to rats. The new finding can be taken as additional support for the theory (7) that lysine is not subjected in the animal to any reversible chemical reactions involving the carbon chain, such as for instance the process of successive deamination and amination of the corresponding keto acid.

² There is, however, one point which makes it difficult to explain our findings directly by that theory. Arginase, supposed to be one of the enzymes involved in urea formation, acts only on free arginine, not on arginine in protein linkage. Our isotopic arginine was isolated from the liver protein. If our finding of the almost equal isotope concentration in the urinary as well as in the "arginine urea" were to be explained by the Krebs theory, it would be necessary to assume either that the arginine in protein linkage is involved in urea formation, or that free arginine and protein arginine continuously exchange their places in the liver.

³ If our findings be explained by the Leuthardt theory, the same assumption would have to be made as for the isotopic arginine: either glutamine and asparagine, while in protein linkage, are involved in urea formation, or free glutamic and aspartic acids exchange their places continuously with the same acids in protein linkage.

The lack of labeled nitrogen in the lysine of the earlier experiment (2) was taken as support of the theory that the introduction of the isotope into other amino acids had not occurred by mere physical exchange of the nitrogen but rather by chemical reaction. The same reasoning applies to the present experiment: the introduction of tyrosine nitrogen into the other amino acids must have occurred by chemical reaction.

Mechanism of Nitrogen Transfer in Amino Acids—The results of the present experiment are similar to that in which labeled ammonia was given to immature rats (2). The distribution of labeled nitrogen in the different fractions of the proteins runs roughly parallel in the two experiments. This might indicate that in the present experiment ammonia was an intermediate between tyrosine and the newly formed compounds. However, in the earlier experiments very large quantities of labeled nitrogen were given (1.4 gm. of N daily per kilo of body weight), whereas in the present study the animal received only 60 mg. of tyrosine N daily per kilo of body weight. If the experiments could be compared, tyrosine would seem to be a much more efficient "nitrogen donor" than ammonia.

The two experiments, however, are not comparable. The first one with ammonia was carried out under unphysiological conditions, while in the present study the conditions were normal. A control experiment in which moderate amounts of ammonia are added to the normal stock diet may reveal the mechanism.

SUMMARY

1. An adult, non-growing rat was kept for 10 days on a stock diet, the tyrosine of which was labeled by the addition of *dl*-tyrosine N¹⁵.

2. Only about half of the labeled nitrogen was recovered in the urine, while almost all of the remainder was found in the tissues. Only a small quantity was located in the non-protein nitrogen of the tissues; the bulk was in the proteins.

3. The liver protein contained about 3 times the concentration of labeled nitrogen found in the proteins of the rest of the body. The proteins were hydrolyzed, and a number of their constituents were isolated to locate the position of the isotope.

4. Tyrosine samples isolated separately from the liver proteins and from the proteins of the rest of the body had a high concentra-

tion of the labeled nitrogen. At least 8 per cent, and probably more, of the total dietary tyrosine was introduced into the animal's proteins. The total amount of isotope in the tyrosine accounted for only 20 to 25 per cent of the total isotope in the proteins.

5. The isotope was also located in (a) the monoaminodicarboxylic acids, (b) histidine, (c) arginine, and (d) the "amide nitrogen." No labeled nitrogen was found in lysine.

6. Other protein constituents not isolated must also have contained labeled nitrogen, as the total amount of isotope in the substances isolated accounted for only a fraction of that in the proteins.

7. The labeled nitrogen in the arginine from the liver protein was located in that part of the guanido group which is split off as urea by arginase. The ornithine moiety of the arginine did not contain labeled nitrogen.

8. Chemical reactions are responsible for the biological formation of the isotopic substances.

9. The experiment indicates that in a healthy, non-growing rat given the ordinary stock diet and kept in nitrogen equilibrium, the nitrogen of at least one dietary amino acid, tyrosine, is only partly excreted in the urine, while most of the remainder is deposited in tissue proteins. An equivalent of protein nitrogen is excreted. Only part of the deposited nitrogen remains attached to the original carbon chain of tyrosine, and a considerable portion of the rest is used for the formation of other nitrogenous compounds, even of such amino acid molecules as were abundant in the diet.

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STUDIES ON ENZYME ACTION

LI. THE PHOSPHATASE ACTIONS OF TISSUES OF NORMAL AND RACHITIC RATS

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In an earlier paper (1), the lipase actions on a number of esters of extracts of tissues of normal and rachitic rats were presented. The actions of the liver and kidney extracts of the normal and the rachitic rats were essentially similar, but with the lung extracts the types or "pictures" of the actions on the esters were not the same. Phosphatase action may be considered as a group of the more inclusive term "lipase action." In this paper, the results obtained in a study of such phosphatase action will be presented.

No attempt will be made to cover the literature pertaining to studies along similar lines. Reference must be made, however, to the recent paper by Bodansky (2) in which the results of such a related study were presented and discussed from various points of view.

EXPERIMENTAL

Enzyme Extracts—The tissues were obtained from (1) rats fed a normal diet, (2) rats made rachitic by being bred from parents fed a deficient diet and then themselves fed Steenbock Ration 2965 (3) for 28 to 32 days. The rats were killed with chloroform; the kidneys, lungs, livers, and hearts of a group of from four to twelve animals were dissected out, ground with sand, and water added in a proportion of 300 cc. to 4 gm. of tissue. Toluene (1 cc. to 100 cc. of mixture) was added; the mixture allowed to stand at room temperature overnight, and filtered through paper. Whole rat extracts were prepared similarly except for the use of a meat grinder in place of grinding with sand. For the enzyme experiments, in order to obtain satisfactory colorimetric readings, 8 cc.

each of liver, lung, heart, and whole rat extracts and 1 cc. portions of kidney extracts were used.

Kay's method (4) with both sodium glycerophosphate and calcium hexosediphosphate¹ at pH 7.6 for 48 hours and Bodansky's method (5) with sodium β -glycerophosphate at pH 8.6 for 1 hour were used. The method described by Kay for plasma was used for the rat tissue extracts.

Results

In Kay's method pH 7.6 was adopted in place of the 8.9 which has been used in a number of investigations. No attempt was made here to attain maximum action for a tissue extract by changing the pH or by adding various substances. A comparison of the phosphatase actions of the various extracts was in view. The study may perhaps be considered from the point of view of the system as a whole rather than as purely enzymic actions. It was, therefore, considered preferable to measure these actions with the materials under conditions of extraction as nearly comparable as feasible rather than modify the actions by added substances. The latter procedure would be a separate study. The estimation of the total nitrogen in the various extracts obtained by the Kjeldahl method are presented in Table I.

The variations in the nitrogen contents of the tissue extracts do not seem unusual. The tissues of the normal and of the rachitic rats behaved similarly in so far as the total nitrogen of the extracts was concerned. The ages of the normal animals (35 to 270 days) exerted no apparent influence; the ages of the rachitic animals ranged from 54 to 61 days.

The results of the phosphatase estimations are presented in Table II. The ages of the normal animals are not given in the groups of the results, since they did not appear to influence the enzyme determinations in any systematic way.

The first fact which appears in these results is the unexpected variation which occurred in the actions of some of the extracts of any one tissue. Each extract was prepared from material from four to twelve rats, so that the possibly exceptional behavior of one animal is ruled out. Attempts were made to maintain the

¹ Thanks are due to Dr. Kenneth C. Blanchard of Washington Square College, New York University, for the calcium hexosediphosphate used in this investigation.

conditions for the living animals, for the preparation of enzyme material, and for the enzyme test as uniform as possible, but no attempts were made to obtain increased or retarded actions or so called optimum actions by the addition of various substances.

In Table III are presented the average values of the actions given in detail in Table II, and in each case in parentheses, the number of experiments upon which that average is based.

The conclusions to be drawn from the results presented in Tables II and III may be given briefly. The absolute values, including the averages and also the ranges, of the actions are much the same for the normal and the rachitic rats. These similar actions are of special interest because of the considerable variation

TABLE I
Nitrogen Contents of Rat Tissue Extracts

Rat tissue	No. of estimations	N per cc. extract	
		Ranges	Averages ($\times 10^2$)
		mg.	mg.
Normal, liver	18	0.11-0.22	17.9
“ kidney	13	0.20-0.28	25.2
“ lung	8	0.19-0.23	20.3
“ whole rat	13	0.11-0.16	14.0
“ heart	1	0.13	13.0
Rachitic, liver	8	0.15-0.25	19.4
“ kidney	6	0.23-0.31	25.8
“ lung	5	0.18-0.24	20.8
“ whole rat	4	0.09-0.19	14.3
“ heart	3	0.10-0.15	12.0

found at times in the sets of results for any one tissue (Table II), but they are unmistakable. The differences observed in two cases, liver and whole rat by the Kay method with sodium glycerophosphate, cannot be stressed because of their small intrinsic values and are possibly more apparent than real. The ratios of the actions by the Kay method on the two phosphoric esters, sodium glycerophosphate to calcium hexosediphosphate, are as follows:

	Kidney	Liver	Whole rat	Heart	Lung
Normal	0.64	0.37	0.65	0.62	0.94
Rachitic	0.64	0.56	0.85	0.60	0.95

The large values for the ratio of the lung are of interest. This signifies that, under the given conditions of testing, the actions of the lung extracts on the two esters were much the same, but that with the other tissues, under these same conditions, the actions on calcium hexosediphosphate were in the neighborhood of 50 per cent greater than on sodium glycerophosphate. The somewhat

TABLE II

Phosphatase Actions of Extracts (4 Gm. per 300 Ml. of Water) of Tissues of Normal and of Rachitic Rats by Kay Method (with Sodium Glycerophosphate and Calcium Hexosediphosphate As Substrates) and by Bodansky Method (with Sodium Glycerophosphate As Substrate)

Tissue	P liberated per ml. of tissue extract					
	Normal rats			Rachitic rats		
	Average	Range	No. of estimations	Average	Range	No. of estimations
Kay method. Substrate, sodium glycerophosphate						
Liver	0.014 ₀	mg.	mg.	0.019 ₈	mg.	mg.
		0.005-0.009	7		0.014-0.019	6
		0.010-0.014	5		0.020-0.024	6
		0.015-0.019	5		0.026	1
		0.020-0.024	3			
Kidney	0.569 ₉	0.026	1	0.550 ₁		
		<0.450	2		<0.450	2
		0.450-0.520	8		0.450-0.500	2
		0.650-0.710	6		0.500-0.540	2
		>0.710	2		0.580-0.650	7
Lung	0.046 ₈			0.042 ₇	>0.750	1
		0.027-0.035	2		0.033-0.035	2
		0.040-0.045	8		0.036-0.045	2
		0.050-0.055	5		0.045-0.047	5
		>0.055	2		>0.052	1
Whole rat	0.024 ₄	0.009-0.014	3	0.032 ₉	0.018	1
		0.015-0.020	5		0.025	1
		0.028-0.038	3		0.035-0.038	6
		0.040-0.048	3			
Heart	0.023 ₀	0.007	1	0.024 ₀	0.006	1
		0.010	1		0.022	1
		0.052	1		0.024-0.028	5
					0.031	1

TABLE II—*Concluded*

Tissue	P liberated per ml. of tissue extract					
	Normal rats			Rachitic rats		
	Average	Range	No. of estimations	Average	Range	No. of estimations
Kay method. Substrate, calcium hexosediphosphate						
Liver	0.037 _s	0.033	3	0.036 _o	0.028	1
		0.036-0.038	7		0.033-0.034	2
		0.039-0.041	7		0.036-0.039	10
		0.045	1			
Kidney	0.895 _s	0.521	1	0.854 _s	0.566	1
		0.640-0.800	5		0.750-0.850	6
		0.800-0.920	7		0.910-0.950	3
		1.000-1.200	4		1.000-1.060	2
Lung	0.049 _s	1.631	1	0.045 _s	0.036	1
		0.045-0.049	6		0.041-0.045	3
		0.050-0.055	8		0.046-0.050	6
		0.057	1		0.036-0.039	5
Whole rat	0.037 _s	0.024	1	0.038 _s	0.036-0.039	5
		0.031-0.043	12		0.040-0.042	3
		0.056	1			
Heart	0.037	0.037	2	0.040 _o	0.036-0.040	5
					0.043-0.046	3
Bodansky method. Substrate, sodium glycerophosphate						
Liver	0.004 _s	0 -0.019	6	0.005 ₇	0.001-0.019	10
Kidney	0.175 _s	0.078	1	0.176 _s	<0.050	2
		0.120-0.200	2		0.100-0.150	2
		0.200-0.235	3		0.230-0.260	6
Lung	0.044 _o	0.030	1	0.042 ₇	0.032-0.037	2
		0.040-0.045	2		0.044-0.049	5
		0.050-0.056	2			
Whole rat	0.025 _s	0.013	1	0.030 ₁	0.012-0.017	2
		0.023-0.024	2		0.033-0.040	5
		0.041	1			
Heart	0.023 _s	0.018-0.027	3	0.027 _o	<0.010	2
					0.025-0.035	5
					0.070	1

lower ratio for normal liver and the higher ratio for rachitic whole rat (less than those for the lung, however) are probably more apparent than real, as already indicated.

The comparative action of the various tissue extracts on the same substrate (sodium glycerophosphate) by the two methods (Kay and Bodansky) are of interest. The amounts of hydrolysis were much the same for the lung, whole rat, and heart extracts. On the other hand, the Kay method gave larger actions than the Bodansky method with liver and kidney extracts. The liver extracts gave only very small actions by the Bodansky method, so that they are of no practical or scientific interest at the present time. As for the kidney extracts, the actions obtained by the

TABLE III

Averages of Phosphatase Actions of Tissues of Normal and of Rachitic Rats Shown in Detail in Table II

		Kay method		Bodansky method, sodium glycerophosphate
		Sodium glycerophosphate	Calcium hexosediphosphate	
		mg.	mg.	mg.
Liver	Normal	0.014(21)	0.038(18)	0.005 (6)
	Rachitic	0.020(13)	0.036(13)	0.006(10)
Lung	Normal	0.046(17)	0.049(15)	0.044 (5)
	Rachitic	0.043(10)	0.045(10)	0.043 (7)
Kidney	Normal	0.570(18)	0.896(18)	0.176 (6)
	Rachitic	0.550(14)	0.854(12)	0.176(10)
Whole rat	Normal	0.024(14)	0.037(14)	0.025 (4)
	Rachitic	0.033 (8)	0.039 (8)	0.030 (7)
Heart	Normal	0.023 (3)	0.037 (2)	0.023 (3)
	Rachitic	0.024 (8)	0.040 (8)	0.027 (8)

The figures in parentheses represent the number of experiments included in the average.

Bodansky method are about one-third of the actions obtained by the Kay method. These actions by the Bodansky method, however, are quite large. In many cases, therefore, for materials such as kidney, etc., the Bodansky method might be preferable because of the shorter time of action and presumably smaller secondary actions. The magnitudes of the actions on calcium hexosediphosphate were in all cases equal to, or greater than, the actions on sodium glycerophosphate, both by the Kay method, under the given conditions of testing.

The results of Kay (6) indicated that the phosphatases of differ-

ent tissues and serum are probably identical, while Bodansky (2) has presented considerable evidence on this point. The latter discussed this phase of the problem in detail and showed differences between intestinal phosphatase and bone and kidney phosphatase when various substances such as taurocholic, etc., acids were added to the enzyme-acting mixtures. The results presented in the present paper do not add directly to this phase of the general problem.

SUMMARY

The phosphatase actions of the lung, liver, kidney, heart, and whole rat extracts of normal and rachitic rats were determined by the Kay method on sodium glycerophosphate and calcium hexosediphosphate and by the Bodansky method on sodium glycerophosphate.

No difference in actions was observed between the normal and rachitic animal preparations.

The relative actions or the ratio of the actions on the two esters under the given conditions differed in the case of the lung extracts from the ratios with extracts of the other tissues both for the normal and the rachitic animals.

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THE ACTION OF VANADIUM ON THE OXIDATION OF PHOSPHOLIPIDS BY CERTAIN TISSUES*

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Vanadium salts can act as catalysts in the oxidation of certain organic compounds, for instance aniline (2). Vanadium is found according to Spiro (3) in most animal tissues and it accumulates in the liver when added to the diet (4). It is therefore possible that the vanadium which is absorbed in small amounts in the food may have a metabolic function. Indications that vanadium may affect metabolism are given in the work of Lyonnet, Guinard, Martz, and Martin (5), Jackson (6), and Martin (7). The effect of vanadium on enzymes has been studied by Neuberg and Kobel (8) who showed that it accelerated the hydrolysis of hexosediphosphoric acid by yeast extracts, and by Lyonnet (9) who demonstrated an inhibiting action on trypsin and pepsin. More recently vanadium has been shown to play a part in the nitrogen assimilation of *Azotobacter* (10). The pharmacology of vanadium salts has been studied by Priestley (11), Jackson (6), Fournier, Levaditi, and Schwarz (12), and Levaditi (13). Henze (14) demonstrated vanadium in certain cells of ascidians. Because of its catalytic action on oxidations it was of interest to try its effect on the oxygen uptake of various tissues.

EXPERIMENTAL

The tissues of the rat and guinea pig were used. They were chopped with scissors, ground with sand in a mortar after the addition of 0.05 M phosphate buffer of pH 6.7, and squeezed through muslin. If 20 to 40 micrograms of vanadium as sodium metavanadate or as vanadium acetate were added to these tissue suspen-

* A preliminary account has appeared (1).

sions, the oxygen uptake of liver was greatly increased, that of kidney slightly increased, and that of brain unaffected. The livers of the rat or guinea pig were therefore used in the following experiments and the effect of vanadium on the oxygen uptake of rat liver is shown in Fig. 1. As there was little or no increase in the carbon dioxide production of the liver, it appeared that vanadium was catalyzing the first stages of the oxidation of some substance in the liver. In order to study the mechanism of this catalysis the following experiments were done.

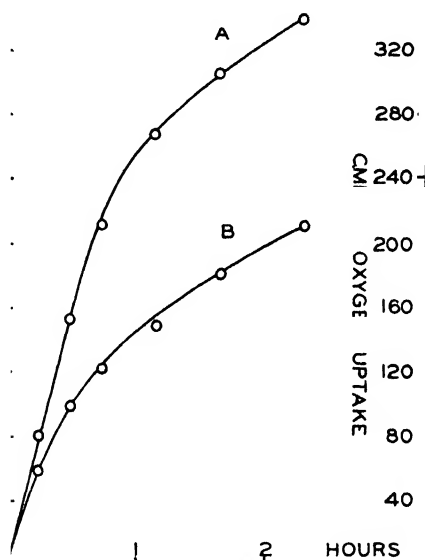


FIG. 1. The oxygen uptake of rat liver suspension at pH 6.7 and 37°. Curve A, with 0.1 mg. of sodium metavanadate; Curve B, control.

When the liver suspension was centrifuged at pH 6.7, the proteins which were insoluble under these conditions could be separated. This insoluble fraction contained a large number of the active oxidative catalysts but because the soluble substrates had been washed away a suspension of this fraction took up very little oxygen. After the first washing the oxygen uptake was only about 25 per cent of that of the original liver suspension and the vanadium effect had diminished correspondingly. After the third or

fourth washing both the oxygen uptake and the vanadium effect had disappeared. If a liver suspension was dialyzed overnight in the ice box, its oxygen uptake was greatly diminished because of the loss of dialyzable substrates but the effect of vanadium was still about 70 per cent as great as in the original fresh liver suspension. These experiments suggest that the substance which is

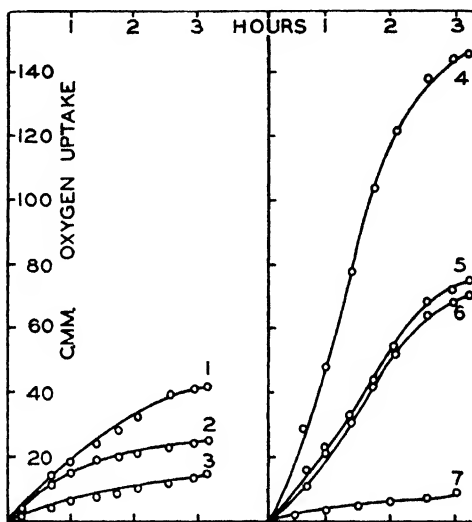


FIG. 2. The oxygen uptake of washed guinea pig liver in the presence of various phospholipids with and without vanadium at pH 6.7 and 37°. The oxygen uptakes of the controls without phospholipids are subtracted from each curve. Curve 1, liver and 5.0 mg. of soy bean lecithin; Curve 2, liver and 2.5 mg. of alcohol extract of guinea pig liver precipitated with acetone; Curve 3, liver and 2.5 mg. of soy bean lecithin; Curve 4, liver, 0.1 mg. of sodium metavanadate, and 5.0 mg. of soy bean lecithin; Curve 5, liver, vanadate, and 2.5 mg. of soy bean lecithin; Curve 6, liver, vanadate, and 2.5 mg. of guinea pig liver extract; Curve 7, vanadate and 5.0 mg. of soy bean lecithin.

oxidized in the presence of vanadium is only slowly dialyzable and that it is probably a large molecule or is attached to a protein which is soluble under the conditions of washing described above.

The washings of the liver suspension were precipitated with 80 per cent alcohol, the proteins filtered off, and the filtrate evaporated free of alcohol. The fatty suspension remaining contained the substrate the oxidation of which was catalyzed by vanadium

in the presence of the liver protein. The suspension was evaporated to dryness, taken up in an alcohol-ether mixture, and precipitated with acetone. The resulting mixture of phospholipids when dissolved in buffer and added to the washed liver protein and vanadium was rapidly oxidized, but added to the protein alone was oxidized slowly. The vanadium alone had little or no effect.

Fig. 2 shows the oxidation of liver phospholipids by washed liver protein with and without vanadium compared to soy bean phospholipids. The latter were supplied by the Glidden Company and precipitated several times from an alcohol-ether mixture by acetone. The liver protein was prepared by washing guinea pig or rat liver suspensions made from 10 gm. of liver four times with 50 cc. of buffer of pH 6.7 and making the final suspension up to 15 cc. with buffer of pH 6.7. The sodium metavanadate was made up in a concentration of 1.0 mg. per cc. Each Warburg vessel contained 0.5 cc. of protein suspension, 0.1 cc. of vanadate solution, a suitable amount of phospholipid, and buffer was added to make the final volume 2.0 cc. Fig. 2 shows that the oxygen uptake was a function of the phospholipid concentration. The uptake was independent within limits of the vanadium and protein concentrations. The oxidation of amino acids, amines, alcohol, aldehyde, lactate, succinate, fumarate, pyruvate, glucose, citrate, glycerol, choline, and the lower fatty acids was not affected by vanadium. Fig. 3 shows the effect of vanadium on the oxidation of soy bean cephalin by washed rat liver. The cephalin fraction of the soy bean phospholipids is oxidized by the liver protein alone more rapidly than the lecithin fraction and the vanadium effect is therefore not so great.

Phospholipids extracted from heart and brain by alcohol-ether mixtures and precipitated by acetone are oxidized. Phospholipids from egg are very slowly oxidized in comparison with those from brain. According to Rae (15) egg contains mostly β -lecithins, whereas brain contains mostly α -lecithins. This suggests that the juxtaposition of the two fatty acids allows the oxidation by the vanadium system to proceed more rapidly. The constituent parts of the lecithin molecule are not attacked under the conditions of these experiments except for certain fatty acids such as oleic, stearic, and palmitic acids, which, although not

oxidized by the liver protein alone, are oxidized very slightly in the presence of the vanadium (Fig. 3, Curve 3). This indicates that the oxidation takes place in the fatty acid part of the phospholipid.

That the fatty acid part of the molecule is attacked is shown in Table I. At the end of the oxidation the proteins were precipitated by 80 per cent alcohol and filtered off. The filtrate was evaporated to dryness and taken up in 10 cc. of chloroform and the double bonds estimated by the method of Yasuda (16). When vanadium is added to the untreated liver suspension, there is an

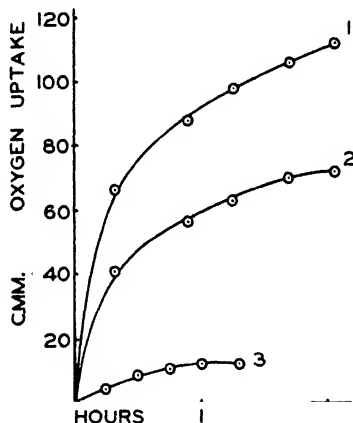


FIG. 3. The oxygen uptake of washed rat liver and 5.0 mg. of soy bean cephalin with (Curve 1) and without (Curve 2) 0.1 mg. of sodium metavanadate. Curve 3, the oxygen uptake of washed guinea pig liver, 0.1 mg. of vanadate, and 5.0 mg. of sodium oleate. Without vanadate the oleate is not oxidized by the liver. The respective controls are subtracted from each curve. pH 6.7 and 37°.

increase in the number of double bonds over that in the control. The vanadium, however, when added to the washed protein and phospholipid caused a marked decrease in double bonds in the added phospholipid. These facts indicate that vanadium is causing both an increased dehydrogenation of the fatty acid and an oxidation of the double bond. In the untreated liver the dehydrogenation predominates; in the washed protein-phospholipid system the oxidation of the double bond predominates. In

both cases probably the change measured is the algebraic sum of the two processes.

If crystallized rat hemoglobin is added to phospholipids at pH 6.7, a definite oxygen uptake occurs. This is greatest for soy bean lecithin, less for soy bean cephalin, and still less for liver lecithin. During this catalytic oxidation of the phospholipids the hemoglobin is rapidly converted to methemoglobin. Vanadium has no effect on this oxidation, which shows that traces of hemoglobin which may remain in the washed liver preparations play no part in the vanadium effect. Moreover, liver and soy bean lecithin are oxidized equally readily by the vanadium-

TABLE I

Change in Relative Number of Double Bonds of Phospholipids after Oxidation in Presence of Guinea Pig Liver and Vanadium

The figures are given in cc. of 0.02 N thiosulfate and are proportional to the number of double bonds present. pH 6.7, 37°.

Tissue	0.02 N thiosulfate
	cc.
0.2 cc. untreated liver suspension.....	1.13
0.2 " " " " + 0.1 mg. vanadate.....	2.58
0.5 " protein suspension.....	2.35
0.5 " " " + 0.1 mg. vanadate.....	2.62
0.5 " " " + liver phospholipid.....	3.75
0.5 " " " + " " + 0.1 mg. vanadate.....	2.27

protein system but the hemoglobin-catalyzed oxidation rates are different.

Oxidation of the soy bean lecithin by the vanadium-protein system is inhibited 70 per cent by 0.0025 M KCN and 90 per cent by 0.005 M KCN. Pyrophosphate in concentrations of 0.25 to 0.5 per cent caused a 20 to 30 per cent, and 1.0 per cent sodium fluoride a 10 to 20 per cent inhibition. 0.0001 M *p*-aminophenol inhibited the oxidation completely. 0.0002 M aminopyrine inhibited 50 per cent, but 10 times this amount of antipyrine had no effect. The oxidation of soy bean lecithin by hemoglobin is accelerated, after a short initial inhibition, by fluoride, which again differentiates the hemoglobin from the vanadium-protein

catalysis. The fluoride acceleration of the former is possibly caused by the formation of fluoride methemoglobin. The vanadium-protein catalysis decreases rapidly with increase of pH, for at pH 7.8 the oxidation rate is only 30 per cent of that at pH 6.7. The catalysis by hemoglobin is increased slightly by increasing the pH from 6.7 to 7.8.

DISCUSSION

Although vanadium is considered a rare metal because of the relatively few commercially workable deposits, it is according to Mellor (17) more abundant in the earth's crust than lead, copper, or nitrogen and is found in most plant and animal tissues in small concentrations. Since it is known to be catalytically active in various oxidations, it is possible that it plays such a rôle in the body, particularly in the liver where it is found in greatest concentration. Under the conditions of the experiments described above the vanadium salts have by themselves little or no catalytic activity. It is only when a suitable protein is also present that they become active in the oxidation of phospholipids but of none of the other substances tried. The fact that the protein preparation without added vanadium shows some ability to oxidize the phospholipids may indicate the presence of small amounts of vanadium still attached to the protein and that vanadium-protein complexes are normally active in this oxidation.

It is interesting that *p*-aminophenol and aminopyrine inhibit the oxygen uptake of liver suspensions much more effectively at pH 6.7 than at 7.8 and that the vanadium effect is greatest at pH 6.7, and that these drugs inhibit the effect of added vanadium. The action of the drugs is much less on the uptake of kidney and brain and the vanadium effect is much less on these organs. These parallelisms suggest that the inhibiting effect of these drugs on the oxygen uptake of tissues may be due to their inactivation of a vanadium-catalyzed oxidation normally functioning in those tissues.

SUMMARY

1. 20 to 40 micrograms of vanadium added as sodium metavanadate or vanadium acetate to rat or guinea pig liver suspension at pH 6.7 increase the oxygen uptake. Under the same con-

ditions the uptake of kidney is slightly increased but that of brain is not affected.

2. If phospholipids are added to a thoroughly washed liver suspension and vanadium, a rapid oxygen uptake occurs. Vanadium alone has no appreciable effect and the suspension alone oxidizes the phospholipid slowly. No other substrate was found to be oxidized by the vanadium-protein system.

3. The oxidation occurs in the fatty acid part of the phospholipid.

4. The properties of the oxidation are described.

5. Hemoglobin catalyzes the oxidation of phospholipids to a certain extent. This catalysis is not affected by vanadium.

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DELPHININE

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The seeds of *Delphinium staphisagria*, L., have been found on extraction to yield an appreciable alkaloid fraction only a portion of which has been obtained in crystalline form. This appears to consist essentially of the alkaloid delphinine. Our knowledge of the chemistry of the latter, as in the case of the alkaloids contained in other *Delphinium* species and the unquestionably closely related aconite alkaloids, is but in the earliest stage. This may be briefly summarized as follows: Walz (1) revised the previous formulations proposed by earlier workers to $C_{34}H_{47}O_9N$ on the basis of the analysis of the alkaloid itself and of its oxalate. This formula has been supported by Keller (2) and by the analyses reported by Markwood (3). The earlier work of Katz (4) had shown the alkaloid to contain methoxyl and to yield benzoic acid on saponification. Subsequently Walz and Keller confirmed the presence of four methoxyl groups and one benzoyl group. On the basis of acyl determinations on an amorphous "acetyl delphinine" and "propionyl delphinine," Walz concluded that at least one acylatable hydroxyl group is present in the alkaloid. Thus, in the four methoxyl groups and the benzoyl and hydroxyl groups, 7 of the 9 oxygen atoms of delphinine appeared to be accounted for.

As a possible aid in the approach to the chemistry of the aconite alkaloids, we have recently included the *Delphinium* alkaloids in our studies, since they appear to contain fewer of the troublesome oxygen atoms. The delphinine prepared by us from commercial *staphisagria* seeds was found to agree essentially with the properties already recorded for this alkaloid. However, our analytical results have been consistently in closer agreement with the figures required by a modified formula, viz. $C_{33}H_{46}O_8N$. This

formula was also supported by the analysis of the *hydrochloride* of delphinine. Our analyses have confirmed the presence of four methoxyl groups in the alkaloid.

A study of the saponification of the alkaloid with alkali and titration of the liberated volatile acids has shown that two acid groups are removed in the process. The liberation of benzoic acid was confirmed but in addition acetic acid was found to be present and was isolated as the silver salt. Experiments which are still in progress, and which will be reported in another connection, have indicated the presence of a hydroxyl group.

Thus the functions of the 9 oxygen atoms of the alkaloid are defined by the four methoxyl groups, two acyl groups, and one hydroxyl group. On catalytic hydrogenation 3 moles of hydrogen were absorbed by the alkaloid and a crystalline *hexahydrodelphinine* resulted. Since the latter on saponification no longer yielded benzoic acid but hexahydrobenzoic acid instead, the alkamine portion of the molecule was not hydrogenated. This parallels the experience with aconitine (5) and suggests a saturated character for the parent alkamine.

In addition to the four methoxyl groups, delphinine has now been found to behave as if it contained an N-alkyl group. When the alkaloid was submitted to alkali fusion, methylamine was isolated from the volatile material and was identified as the picrate. If the group in question is an N-alkyl group, it must therefore be methyl. Hence there is a close parallelism in the pictures presented by delphinine and the aconite alkaloids. In the case of the latter, aconitine $C_{34}H_{47}O_{11}N$ (6, 7) behaves as if an N-ethyl group is present in addition to four methoxyl groups, three hydroxyl groups, a benzoyl, and an acetyl group. In hypaconitine, $C_{33}H_{46}O_{10}N$, and mesaconitine, $C_{33}H_{46}O_{11}N$, the N-alkyl group has been shown to be methyl (7).

Another observation which parallels the experience with aconitine is the so called "X-214°," a substance obtained by Keller (2) by the oxidation of delphinine with permanganate and in which the salt-forming properties of delphinine have been lost. A provisional formula of $C_{28}H_{37}O_9N$ was derived for it. We have confirmed the production of this substance and the possibility is at once suggested of its analogy to oxonitine from aconitine. This is supported by the fact that just as oxonitine no longer contains the N-ethyl group (or equivalent) of aconitine, this oxi-

dation product likewise no longer possesses the N-methyl group (or equivalent) of delphinine. Titration after saponification has shown the liberation of two acid groups and therefore the retention of the acetyl and benzoyl groups of delphinine, just as it has been found to be the case with oxonitine. It is most likely a lactam. A discussion of the exact formulation of this substance we wish to leave to a later occasion.

EXPERIMENTAL

Delphinine—The alkaloid fraction was obtained from the powdered seeds essentially as described by Markwood. The concentrated ligroin extract of the seeds was in turn exhaustively extracted with 3 per cent tartaric acid. During the first few extractions with the latter, calcium tartrate crystallized during the process and was removed by filtration. Later extractions were not complicated by this crystallization and the process was continued as long as the acid extract gave a precipitate with alkali. The acid extracts were cleared by shaking out successively with petroleum ether and ether and were then made alkaline. The precipitated alkaloid was reextracted with ether. The latter on concentration gave a copious basic oil in which crystals of delphinine formed. After standing the alkaloid was collected with a mixture of ether and petroleum ether. The yield approximated 0.8 gm. from 1 kilo of seeds. After recrystallization from alcohol the alkaloid formed six-sided plates which melted at 198–200°. Keller and Walz, as well as Markwood, have given the uncorrected melting point of 187.5°, and Stojanow the corrected figure 191.8°. We have found $[\alpha]_D^{25} = +25^\circ$ ($c = 1.215$ in absolute alcohol). Keller gave $[\alpha]_D^{20} = +18.99^\circ$ for a solution which had stood for 4 hours. He also reported a rotation taken shortly after solution of the alkaloid from which can be calculated a rotation of $[\alpha]_D^{20} = +22^\circ$ ($c = 0.86$ in absolute alcohol). This value is comparable with our own.

$C_{22}H_{26}O_2N$.	Calculated.	C 66.07, H 7.57, N 2.34
$C_{21}H_{25}O_2N$.	"	" 66.52, " 7.72, " 2.28
	Found. (a)	" 66.04, " 7.52
	" (b)	" 66.00, " 7.61
	" (c)	" 65.94, " 7.61
$C_{22}H_{26}O_2N$.	Calculated.	4(OCH ₃) 20.71, (N)CH ₃ 2.51
	Found.	" 20.58
	"	" 20.45, " 2.62

15.295 mg. of delphinine were refluxed in 3 cc. of 0.1 N NaOH and 3 cc. of alcohol for 3 hours and titrated back against phenolphthalein. Found, 0.347 cc. Calculated for 2 equivalents, 0.51.

15.650 mg. were refluxed as above, but for 7 hours. Found, 0.345 cc. Calculated for 2 equivalents, 0.522.

Since the correct titration of the base was undoubtedly affected by the basicity of the alkaline cleaved during the saponification, the following procedure was used.

0.4255 gm. of delphinine was refluxed in a mixture of 10 cc. of N NaOH and 10 cc. of methanol for 2.5 hours and protected as usual from the air. The mixture was acidified with 5 cc. of 25 per cent H_2SO_4 and then distilled with steam. The distillate was titrated with 0.1 N NaOH and was collected as long as the distillate consumed alkali. The total alkali used was 13.66 cc. Calculated for 2 equivalents, 13.88 cc.

The titration mixture was concentrated *in vacuo* and in a volume of about 2 cc. was acidified with dilute HNO_3 . Precipitated benzoic acid was collected with water. After recrystallization from petroleum ether it melted at 121° .

$C_7H_6O_2$. Calculated, C 68.82, H 4.96; found, C 68.70, H 5.02

The above filtrate from the crude benzoic acid was extracted several times with small amounts of petroleum ether to remove dissolved benzoic acid and then carefully neutralized with dilute NaOH. After concentration to a small volume the mixture was treated with strong silver nitrate solution. The collected silver salt was recrystallized from dilute alcohol.

$C_2H_3O_2Ag$. Calculated. C 14.38, H 1.81, Ag 64.64
Found. " 14.62, " 1.89, " 64.72

Delphinine Hydrochloride—The salt was readily obtained by addition of dry ether to the concentrated solution of the alkaloid in methanol containing an excess of HCl. It formed needles from methanol and ether, which melted at $208-210^\circ$, depending upon the rate of heating.

For analysis it was dried at 120° and 15 mm.

$C_{33}H_{46}O_9N \cdot HCl$. Calculated. C 62.28, H 7.29
Found. " 62.16, " 7.47
" " 62.40, " 7.68

Hexahydrodelphinine—0.11 gm. of delphinine was dissolved in a few cc. of alcohol containing 0.2 cc. of acetic acid, and hydrogenated with 50 mg. of platinum oxide catalyst under a pressure of 3 atmospheres. Absorption was prompt and stopped after about 30 minutes. The absorption due to the substance was 12.6 cc. of H_2 or approximately 3 moles. After removal of the solvent the substance crystallized readily under ether. After recrystallization from this solvent it melted at 192–193°.

$C_{11}H_{11}O_2N$. Calculated, C 65.41, H 8.49; found, C 65.33, H 8.21

On saponification this substance gave no benzoic acid but an oily acid which from its odor and properties was unquestionably hexahydrobenzoic acid.

Alkali Fusion of Delphinine—A mixture of 1 gm. of the alkaloid with 5 gm. of powdered KOH was heated in a Pyrex cylindrical vessel immersed in a nitrate bath. Hydrogen was passed through during the operation and volatile material was collected in a bent tube which formed the outlet. The gases were continued through wash bottles containing dilute HCl. Copious fumes were evolved and a yellow, viscous resin began to distil with the bath at 260°. The operation was held at this point for 30 minutes and was then discontinued. The yellowish resin was in large part dissolved by dilute HCl which rapidly assumed a violet color. This mixture was joined with the aid of alcohol with the contents of the wash bottles and was then extracted repeatedly with ether. The aqueous phase was concentrated *in vacuo* to dryness, leaving a partly crystalline residue. On addition of a little water a resin remained, which was removed by filtration. The filtrate and washings were made alkaline and distilled with steam into dilute HCl. The latter on evaporation gave 9 mg. of a crystalline residue.

This salt was decomposed under ether with NaOH. The ethereal solution which smelled strongly of methylamine was dried over KOH. On addition of ethereal picric acid solution a deposit of needles at once formed. After collection it was recrystallized from alcohol ether and melted at 205–207°.

$C_7H_5O_7N_4$. Calculated, C 32.31, H 3.11; found, C 32.41, H 3.01

Oxidation of Delphinine—1.05 gm. of the alkaloid dissolved in 100 cc. of dry acetone were treated with 1.1 gm. of $KMnO_4$ and

the mixture was occasionally shaken. Several days at room temperature were required before the reagent was used up. Since crystals of the oxidation product appeared on the walls of the vessel, more solvent was added and the mixture was warmed to dissolve them. After filtration the MnO_2 was again extracted with warm solvent. On concentration of the filtrate to small bulk successive fractions of the product were obtained, which amounted to 0.49 gm.

For purification it was dissolved in chloroform and shaken out with dilute H_2SO_4 to remove any traces of unchanged alkaloid or other basic material. The concentrated solvent readily crystallized on addition of alcohol. Flat needles separated, which melted at $218\text{--}220^\circ$. Occasionally a melting point of 225° was observed. The apparently neutral character of the substance was confirmed.

For analysis the substance was dried at 100° and 15 mm.

Found.	C 64.58,	H 7.00,	OCH_3 19.60,	$(\text{N})\text{CH}_3$ 0.64
"	" 64.70,	" 6.90		
"	" 64.82,	" 7.04,	" 19.38,	" 0.63

Titration showed the retention of the benzoyl and acetyl groups in this substance.

14.285 gm. of substance were refluxed in 3 cc. of 0.1 N NaOH and 3 cc. of alcohol for 4 hours and then titrated back against phenolphthalein. Found, 0.438 cc. For an approximate molecular weight of 600 the calculated value for 2 equivalents is 0.47 cc.

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STUDIES ON β -GLUCURONIDASE*

I. A METHOD OF PREPARATION AND PURIFICATION

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The preparation of an extract from animal tissues containing an enzyme specific for the hydrolysis of β -glucuronides was first described by Masamune (1). Oshima (2, 3) later studied the distribution of this enzyme in the tissues of the dog and the ox and improved the method of preparation of active extracts.

At the suggestion of Professor G. F. Marrian an investigation was undertaken to determine whether this enzyme might be concerned with the synthesis of estriol glucuronide in the female organism. Furthermore, it was suggested that the hydrolysis of conjugated sex hormones in human urine for assay purposes by a potent preparation of the enzyme might prove to be more efficient than hydrolysis by mineral acids.

As a preliminary to the main problems in this investigation, a study of the methods of preparation and the properties of β -glucuronidase has been made. The present paper deals mainly with a description of the methods of preparation of the enzyme. Further, experiments designed to indicate the possible rôle of the enzyme in the detoxification of glucuronogenic substances by the mammalian organism are now in progress and will be reported at a later date.

Method of Assaying Activity of β -Glucuronidase Preparations

Determination of Glucuronic Acid—The method developed by Miller and Van Slyke (4) for the microdetermination of blood sugar was modified for the determination of glucuronic acid. In

* The term β -glucuronidase is here used in preference to the term β -glucuronosidase as suggested by Masamune.

the blood sugar method, potassium ferricyanide is reduced by the sugar and the amount of ferrocyanide so produced is then determined by a direct titration with standard ceric sulfate solution, setopaline C being used as the oxidation-reduction indicator.

The routine assay of the activity of β -glucuronidase preparations was greatly simplified by substituting the ceric sulfate titration method for the Hagedorn-Jensen technique, as employed by the Japanese workers.

Glucuron, rather than glucuronic acid, was selected for the standardization, since it can be more readily prepared in a pure state. Assuming that the reducing powers of glucuron and glucuronic acid are proportional to their respective molecular weights,¹ it is possible, after titrating known amounts of glucuron with ceric sulfate, to construct a standard graph correlating the corresponding amounts of glucuronic acid with the titration values. The ceric sulfate solution was therefore standardized against glucuron in the following manner.

50 mg. of pure glucuron were dissolved in 50 cc. of distilled water. Aliquots of this solution were then pipetted into 50 cc. boiling tubes, an excess (2.5 cc. per mg. of glucuron) of alkaline potassium ferricyanide solution (5 gm. per liter) was added, and the volumes were equalized with distilled water. The tubes were covered with blown glass bulbs and placed in a vigorously boiling water bath for 15 minutes. They were then cooled in running water and the contents of each tube were titrated² with 0.0216 N ceric sulfate solution³ after the addition of 1 cc. of 18 N H₂SO₄ and 8 to 10 drops of setopaline C solution.

The amounts of glucuronic acid corresponding to the amounts of glucuron used were plotted, as in Fig. 1, against the ceric sulfate titration values.

By using a greater dilution of ceric sulfate, the method was applicable to the determination of much smaller amounts of glucuronic acid. For example, with 0.002 N ceric sulfate solution,

¹ In view of the observation of Goebel and Babers (5) that there is a slight difference in the relative reducing powers of glucuronic acid and glucuron when determined by the Shaffer-Hartmann procedure, this assumption may be only approximately true.

² A standard 10 cc. burette, graduated in 0.05 of a cc., was employed.

³ In the preparation and standardization of the ceric sulfate solution, the directions of Miller and Van Slyke were followed.

0.1 to 1.2 mg. of glucuronic acid could be estimated over a titration range of 14 cc.

Preparation and Treatment of Enzyme Digests—Sodium menthol glucuronide was the substrate used in the experiments described in this paper. Pure menthol glucuronide was prepared by Bang's method, as described by Quick (6). In preparing standard solutions of the sodium salt, the calculated volume of *N* sodium hydroxide solution was added to a weighed amount of menthol glucuronide in a 100 cc. volumetric flask together with 75 cc. of distilled

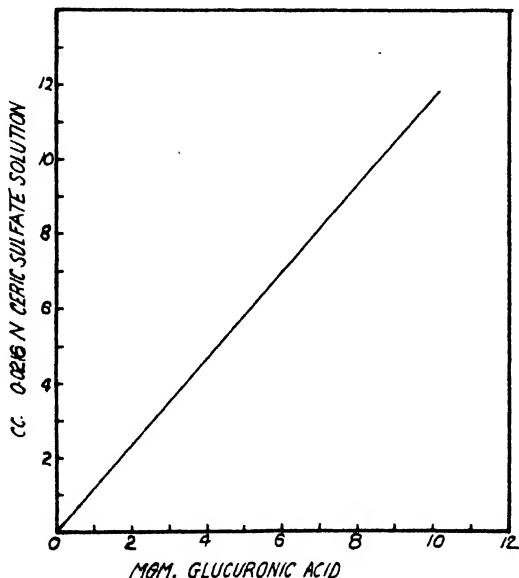


FIG. 1. Standardization curve for glucuronic acid

water. The pH of this solution was then adjusted to 4.8 to 5.4 (outside indicator) with a few drops of *N* acetic acid. The solution was finally diluted to 100 cc.

The experimental tubes (50 cc.) contained enzyme extract, 0.10 *N* acetate buffer of pH 5.2 to 5.4, and substrate solution usually in the ratio of 1:2:1, while the control tube contained buffer, substrate, and boiled enzyme in the same proportions. The tubes were stoppered and incubated for the desired length of time at 37.5°. At the end of this period, each digest was treated in the following manner.

After the digest was transferred to a 15 cc. centrifuge tube, an equal volume of 10 per cent trichloroacetic acid solution was added. Following centrifugation, the clear supernatant solution was returned to the original boiling tube and neutralized to phenolphthalein with 2 N NaOH. Potassium ferricyanide solution (10 cc.) was added and the reducing power determined by the modified ceric sulfate method. The amount of glucuronic acid liberated was read from the standardization curve (Fig. 1) by substituting the titration difference between control and experimental digests.

Preparation and Purification of β -Glucuronidase Extracts

Little success was realized in obtaining a potent purified enzyme extract by the procedure of Oshima (3). After a critical examination of the properties and solubilities of the enzyme, the present method was devised and found to be satisfactory. This process involves (1) initial extraction of the minced tissue with water, (2) acetone precipitation of the crude extract and subsequent extraction of the precipitate so produced with water, (3) acidification of this aqueous extract with acetic acid to pH 4.8 to 5.0, (4) evaporation to small volume in a current of air, (5) the application of an ammonium sulfate fractional extraction and precipitation technique to the concentrate. As high as 140-fold purifications have been achieved in this manner.

The resulting purified solution of the enzyme possessed as great a potency as seemed necessary for the purposes outlined in the introduction and accordingly no further purification studies were undertaken. The details of the method of preparation and purification are as follows:

Fresh beef spleen, free of fascia, was ground in a mincer. 585 gm. of the tissue were then stirred for an hour with 1170 cc. of distilled water. This crude extract (A) was shaken with diatomaceous earth (20 gm. per liter) and centrifuged for 30 minutes. The supernatant solution (B) was precipitated with 2 volumes of acetone. The reddish brown precipitate which formed was centrifuged immediately, the supernatant solution was poured off, and the residue transferred with the aid of a rubber-tipped glass rod to a 2 liter beaker. Inasmuch as acetone seemed to inactivate the enzyme, a 5 minute period of rapid centrifugation, followed by

the removal of some of the acetone from the precipitate with a stream of air, was adopted as a precautionary step at this stage of the purification. The precipitate was vigorously stirred for 1 hour with 1100 cc. of water.

The water-insoluble material was centrifuged off, leaving a very turbid supernatant solution containing the enzyme (C). The solution was adjusted to pH 4.8 to 5.0 with *N* acetic acid and warmed at 37.5° for 20 minutes. It was then centrifuged and the supernatant solution adjusted to pH 7 with *N* NaOH (D). The extract (1100 cc.) was poured into shallow porcelain dishes and evaporated to 160 cc. in a current of air at room temperature (E).

An equal volume of saturated ammonium sulfate solution was added to the concentrate; the precipitate was separated and dissolved in 100 cc. of water (F). The precipitate formed by adding to solution (F) 100 cc. of saturated aqueous ammonium sulfate was then stirred with 350 cc. of 35 per cent saturated ammonium sulfate solution. The insoluble fraction of this mixture was separated by centrifugation and preserved. The addition of 75 cc. of saturated ammonium sulfate solution to the centrifugate caused complete precipitation of the enzyme, which was separated and dissolved in 50 cc. of water. The enzyme was precipitated from this aqueous solution (G) by half saturation with ammonium sulfate and the precipitate was stirred with 300 cc. of 40 per cent saturated ammonium sulfate solution. The insoluble residue was preserved and the extract was then brought to 50 per cent saturation by the addition of 55 cc. of saturated ammonium sulfate solution. The precipitate, after centrifuging, was dissolved in 25 cc. of water (H₁).

An aqueous suspension (130 cc.) of the combined 35 and 40 per cent insoluble material was half saturated with ammonium sulfate and centrifuged. The precipitate was extracted with 300 cc. of 37 per cent saturated ammonium sulfate solution and to the supernatant solution, after centrifuging, 85 cc. of saturated ammonium sulfate solution were added. The precipitate was centrifuged off and dissolved in 25 cc. of water (H₂). Solutions H₁ and H₂ were combined, this final clear solution being denoted as (I) in Table I.

The activity and protein nitrogen content of the extract at each stage of the purification were determined in order to obtain quanti-

tative purification data (Table I). Digests contained 1 cc. of enzyme extract (boiled in the control digests), 2 cc. of acetate buffer at pH 5.4, and 1 cc. of 0.056 N sodium menthol glucuronide. After incubation for 8 hours at 37.5° the liberated glucuronic acid was determined by the ceric sulfate titration method.

TABLE I

Preparation and Purification of β -Glucuronidase from Ox Spleen

Hydrolysis was carried out at pH 5.4, 37.5°, for 8 hours; substrate, 0.0156 N sodium menthol glucuronide.

Step	Stage of preparation	Activ- ity*	Vol- ume of ex- tract	Total activ- ity†	Activ- ity per mg. pro- tein N‡
		mg.	cc.	mg.	mg.
A	Aqueous suspension of minced tissue	4.90	1685	8200	0.63
B	Supernatant solution after centrifuging (A)	4.60	1140	5200	0.66
C	Aqueous extract of acetone ppt. of (B)	3.45	1190	4100	2.41
D	Acidified extract	3.25	1100	3500	8.10
E	Evaporated concentrate	15.5	160	2500	5.96
F	Solution of ppt. obtained from (E) by half saturation with $(\text{NH}_4)_2\text{SO}_4$	20.0	112	2200	16.80
G	Product of fractional extraction of ppt. salted-out from solution (F) with 35% saturated $(\text{NH}_4)_2\text{SO}_4$ solution	29.6	50	1500	45.50
H ₁	(G) repeated on solution (G) with 40% saturated $(\text{NH}_4)_2\text{SO}_4$	34.0	25	850	63.00
I	Solution (H ₁) combined with product of fractional extraction (37%) of insoluble residues from (G) and (H ₁)	26.0	50	1300	87.00

* Mg. of glucuronic acid liberated per 1 cc. of extract.

† Number of cc. of extract times the number of mg. of glucuronic acid liberated per 1 cc. of extract.

‡ Mg. of glucuronic acid liberated per mg. of nitrogen precipitable by 10 per cent trichloroacetic acid.

Before the activity of highly concentrated glucuronidase solutions was assayed, they were first diluted to strengths such as 1:5, 1:10, and 1:20, depending on the amount of enzyme present. This course was necessary to avoid (1) a lowering of the pH of the digest by too great a liberation of glucuronic acid and (2) marked varia-

tions in the velocity of hydrolysis, the latter being eliminated when conditions were such that the final substrate concentrations in the digests were approximately the same. The activity of an extract was found to be directly proportional to the concentration of the enzyme.

In determining the protein nitrogen of the extracts, 1 cc. of the extract was diluted to 2 cc. with distilled water in a 15 cc. centrifuge cup and 2 cc. of 10 per cent trichloroacetic acid were added. After centrifuging, the supernatant solution was discarded, and the precipitate stirred with 5 cc. of water and centrifuged. Absence of sulfate ion indicated the removal of ammonium sulfate from the trichloroacetate precipitate likely to be adsorbed from extracts at steps (F) to (I). After the supernatant solution was discarded, 2 cc. of concentrated sulfuric acid were pipetted into the vessel and the mixture, after standing for 5 minutes, was transferred to a micro-Kjeldahl flask; washings were added and the total nitrogen was determined by a standard micromethod.

An experiment identical with that just described yielded a final preparation whose activity per mg. of nitrogen was appreciably greater than that of preparation (I) (Table I).

SUMMARY

1. A ceric sulfate titration method has been applied to the estimation of glucuronic acid liberated by the hydrolytic action of β -glucuronidase.

2. A rapid method for the concentration and purification of β -glucuronidase from ox spleen is described.

The author wishes to make grateful acknowledgment to Dr. G. F. Marrian, under whose direction this investigation has been carried out, and also to Dr. A. M. Wynne, for much helpful advice.

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THE INFLUENCE OF OPTICAL ISOMERISM ON THE UTILIZATION OF TRYPTOPHANE, HISTIDINE, AND LYSINE FOR GROWTH IN THE MOUSE*

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Interesting differences have been noted in the influence of optical isomerism on the utilization of the various essential amino acids by the rat for growth. Thus, when *d*(+)-tryptophane is added to a diet otherwise deficient in tryptophane it promotes growth which is quite as rapid as that induced when *l*(-)-tryptophane is supplied (3, 21); under analogous conditions *d*(+)-histidine is somewhat less efficient than its natural isomer (9) and *d*(-)-lysine has no stimulating effect (5). The isomers of other essential amino acids have been similarly tested (19).

In a recent paper Kotake, Ichihara, and Nakata (16) imply that marked differences exist between the rat and the mouse in the utilization of *d*(+)-tryptophane;¹ from their presentation one is led to assume (although they do not specifically so state) that *d*(+)-tryptophane is entirely unavailable to the mouse for growth purposes. However, their experimental evidence is far from convincing; certainly their data cannot support any very definite conclusion. Their use of 10 per cent gelatin together with cystine and histidine as the sole sources of dietary nitrogen in their tryptophane-deficient diet was most unfortunate. In the first place,

* The experimental data are taken from a dissertation submitted by John R. Totter in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa.

A report was presented before the American Society of Biological Chemists at Baltimore, March 31, 1938 (*Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **123**, p. cxxii (1938)).

¹ The paper also includes studies on *l*(+)- and *dl*-indolelactic acids and on *l*(-)-methionine and *l*(-)-cystine.

this percentage of protein in the diet was probably too low (1). Because of the shorter life cycle of the mouse and its more intensive metabolism its need of protein would be at least as great as, if not greater than, that of the rat; most diets for the latter contain 15 per cent of protein or more. In the second place, the amino acid deficiencies in gelatin are probably not limited to tryptophane, cystine, and histidine; at any rate gelatin is a notoriously poor protein and will not induce good growth in the rat, even when fed at high levels and supplemented with amino acid mixtures more extensive than this (15).

The Japanese workers also reduced the allotment of salt mixture to half the amount ordinarily used for rats instead of increasing it as recommended by Beard for mice (1). Most of their supplements of tryptophane amounted to 0.05 per cent of the diet; in only two instances were they as great as 0.1 per cent. For the rat even 0.1 per cent is probably suboptimum.

Obviously, if satisfactory comparisons are to be made between *d*(+)- and *l*(-)-tryptophane, or any other pair of amino acid isomers, suitable basic criteria must be established. This was not done. No attempt was made to determine the response of mice to the unsupplemented diet, and growth in the *l*(-)-tryptophane controls was too erratic to constitute a reasonable basis for comparison. The wide variations in weight changes (from a single gain as high as 2.5 gm. in 22 days to a loss as great as 4 gm. in 26 days) indicate clearly that the deficiency of the basal diet was not met by the *l*(-)-tryptophane supplied, whether because the allotment of this amino acid was too low to elicit consistent response or because the basal diet was too inadequate in other respects.

Since the question raised by the Japanese authors is a fundamental one, we have repeated their work on tryptophane, using the tryptophane-deficient casein hydrolysate commonly employed for rats at a level of 15 per cent of the diet (which preliminary tests indicated induced fairly good growth in mice), increasing the Hawk and Oser salt mixture to 8 per cent (equivalent to the 7 per cent of the Osborne-Mendel mixture recommended by Beard), and including control animals on a diet unsupplemented with tryptophane. In addition we have made similar comparisons between the isomers of histidine and lysine.

EXPERIMENTAL

The *l*(-)-tryptophane was prepared from tryptic digests of casein by the method of Cox and King (10); the *l*(-)-histidine and the *l*(+)-lysine were isolated from blood meal hydrolysates by the procedure described by Cox, King, and Berg (11).

The *d*(+)-tryptophane, which was obtained by resolution of *dl*-tryptophane as directed by Berg (2), showed $[\alpha]_D^{20} = +32.4^\circ$ for a 0.5 per cent solution in water. In preparing the *d*(+)-histidine monohydrochloride, *dl*-histidine was first made by heating a concentrated solution of *l*(-)-histidine monohydrochloride in water for 3 hours at 160-165°, as suggested by Duschinsky (12), and removing the hydrochloric acid with silver hydroxide. The *dl*-histidine was combined with *d*(+)-tartaric acid to form the hydrogen tartrate salt. This was subsequently fractionated essentially as directed by Pyman (18). The tartaric acid was removed from the purified tartrate of *d*(+)-histidine by precipitation and the histidine was isolated as the dihydrochloride (7). The latter was converted to the monohydrochloride by the aniline method (8) and this was reprecipitated several times from aqueous solution with alcohol. An 8 per cent solution of the final product in water containing 1 equivalent of hydrochloric acid showed $[\alpha]_D^{20} = -8.24^\circ$. This agrees well with the specific rotations recorded in the literature for *d*(+)-histidine monohydrochloride (see Cox and Berg (9)). The *d*(-)-lysine dihydrochloride was prepared from the *dl* modification which had been synthesized from cyclohexanone by the method of Eck and Marvel (13). Its isolation after resolution with camphoric acid has been previously described (4); $[\alpha]_D^{20}$ for a 3 per cent solution in water was -15.63° .

The basal diets used are indicated in Table I. The tryptophane-deficient casein hydrolysate was prepared according to the method of Berg and Rose (6), the histidine-deficient digest as outlined by Conrad and Berg (7). The zein was a purified product obtained from the A. E. Staley Company of Decatur, Illinois. The *l*(-)-cystine was prepared from hair by a modification of Okabe's method (17).

In each instance when the natural and unnatural isomers of the deficient amino acids were supplied, they replaced an equal weight of the protein or of the basal amino acid mixture. The trypto-

phane supplements were 0.2 and 0.1 per cent, the histidine monohydrochloride 0.5 per cent, and the lysine dihydrochloride 0.75 per cent. When the amino acid was added as the hydrochloride, sodium bicarbonate equivalent to the hydrochloric acid was also included. The vitamin B complex was furnished as 150 mg. of Harris yeast vitamin concentrate in two doses daily. All of the dietary regimens were thus very similar to those usually employed for rats.

The albino mice were of Strain A. Their breeding stock came from the Jackson Memorial Laboratories at Bar Harbor, Maine.

TABLE I
Composition (in Gm.) of Basal Diets

	Trypto- phane- deficient	Histidine- deficient	Lysine- deficient
Tryptophane-deficient casein hydrolysate...	14.7		
Histidine-deficient casein digest.....		14.5	
Whole zein.....			14.2
Histidine monohydrochloride.....			0.4
Cystine.....	0.3	0.3	0.3
Tryptophane.....		0.2	0.2
Agar.....	2.0	2.0	2.0
Salt mixture (Hawk and Oser (14)).....	8.0	8.0	8.0
Sugar.....	15.0	15.0	15.0
Starch.....	36.0	36.0	36.0
Cod liver oil.....	5.0	5.0	5.0
Crisco.....	19.0	19.0	19.0
Sodium bicarbonate.....			0.16
	100.0	100.0	100.26

Usually two or three litters of approximately the same age were used in a single study and were placed on the experimental diet shortly after weaning. Initial weights ranged from 8 to 16 gm. The housing and care were essentially the same as for rats in similar studies. Precautions were taken to avoid exposure to drafts. Weight and food consumption data were recorded every 4 days. Since the actively growing period of the mouse is much shorter than that of the rat (Thompson and Mendel (20)) no advantage is gained by prolonging such studies on mice beyond 40 days.

Data on growth are recorded in Charts I to III, which are

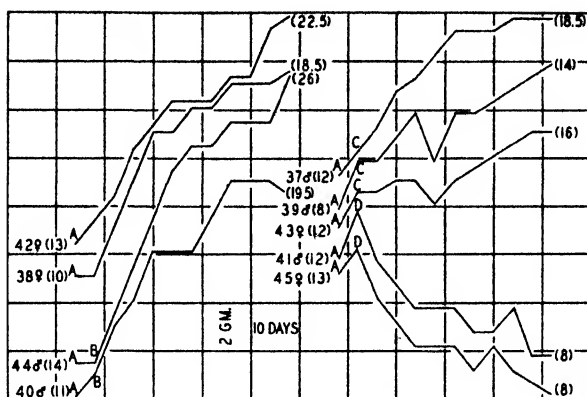


CHART I. Growth curves of mice on a tryptophane-deficient basal diet supplemented with *d*(+)- or *l*(-)-tryptophane. A indicates supplementation with 0.2 per cent of *l*(-)-tryptophane; B, with 0.1 per cent of *l*(-)-tryptophane; C, with 0.2 per cent of *d*(+)-tryptophane. D represents the unsupplemented basal diet. Initial and final weights are given in parentheses.

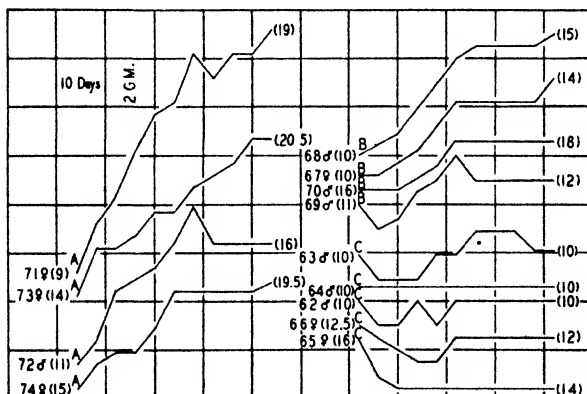


CHART II. Growth curves of mice on a histidine-deficient basal diet supplemented with *d*(+)- or *l*(-)-histidine. A indicates supplementation with 0.5 per cent of *l*(-)-histidine monohydrochloride, and B, with 0.5 per cent of *d*(+)-histidine monohydrochloride. C represents the unsupplemented basal diet. Initial and final weights are given in parentheses.

plotted on the same scale as that used by the Japanese workers. In Chart I comparisons between the animals receiving a supplement of 0.2 per cent of *d*(+)-tryptophane and the controls whose diets contained none show conclusively that this unnatural isomer can support growth. Obviously, however, 0.2 per cent is less efficient than even 0.1 per cent of the natural form. The histidine-deficient diet contained enough histidine to allow maintenance (Chart II). Supplements of *d*(+)-histidine monohydrochloride made growth possible, but at a rate inferior to that attained when the monohydrochloride of natural histidine was substituted. In

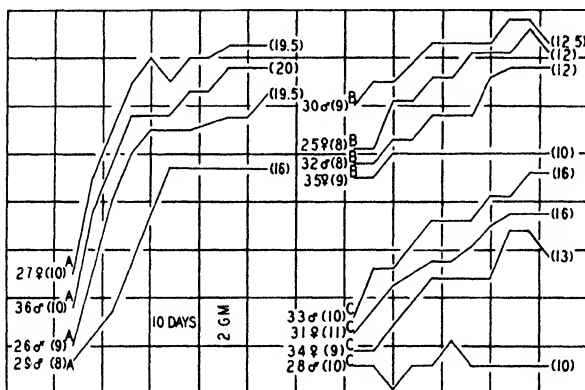


CHART III. Growth curves of mice on a lysine-deficient basal diet supplemented with *d*(-)- or *l*(+)-lysine dihydrochloride. A indicates supplementation with 0.75 per cent of *l*(+)-lysine dihydrochloride; B, with 0.75 per cent of *d*(-)-lysine dihydrochloride. C represents the unsupplemented basal diet. Initial and final weights are given in parentheses.

contrast to these findings are the observations on the rat that the two isomers of tryptophane promote growth with equal efficiency (3) and that *d*(+)-histidine is only slightly inferior to the natural *l*(-)-form (9). The growth obtained in the mouse on *d*(+)-tryptophane and *d*(+)-histidine cannot possibly be ascribed to an admixture of traces of the natural isomers of these amino acids. The specific rotations were in both cases closely in agreement numerically (opposite in direction) with the values recorded in the literature for the natural isomer; even the most generous calculations do not warrant the assumption that enough of the

natural isomer was present to make an appreciable contribution toward growth. The results reported here have been duplicated on a second series of mice. The lysine-deficient diet allowed maintenance or slow growth (Chart III). The diet was not further improved by adding *d*(-)-lysine, but marked growth did occur when

TABLE II
Food Consumption Data for 40 Day Supplementary Period

Mouse No. and sex	Average daily food consumption	Mouse No. and sex	Average daily food consumption	Basal diet deficient in	Supplement
	gm.		gm.		per cent
45 ♀	1.85	41 ♂	1.88	Tryptophane	None
43 ♀	2.35	39 ♂	2.33	"	Tryptophane (0.2 <i>d</i> (+))
		37 ♂	2.40	"	" (0.2 ")
42 ♀	2.51	38 ♀	2.50	"	" (0.2 <i>l</i> (-))
44 ♂	2.93	40 ♂	2.58	"	" (0.1 ")
62 ♂	2.08	63 ♂	2.13	Histidine	None
65 ♀	2.23	64 ♂	2.13	"	"
66 ♀	2.00			"	"
67 ♀	2.25	68 ♂	2.43	"	Histidine monohydrochloride (0.5 <i>d</i> (+))
69 ♂	2.15	70 ♂	2.53	"	Histidine monohydrochloride (0.5 <i>d</i> (+))
71 ♀	2.50	72 ♂	2.58	"	Histidine monohydrochloride (0.5 <i>l</i> (-))
73 ♀	2.88	74 ♀	2.45	"	Histidine monohydrochloride (0.5 <i>l</i> (-))
31 ♀	2.08	28 ♂	1.83	Lysine	None
34 ♀	1.90	33 ♂	1.93	"	"
25 ♀	2.25	30 ♂	1.93	"	Lysine dihydrochloride (0.75 <i>d</i> (-))
35 ♀	2.00	32 ♂	1.83	"	Lysine dihydrochloride (0.75 <i>d</i> (-))
27 ♀	2.38	26 ♂	2.55	"	Lysine dihydrochloride (0.75 <i>l</i> (+))
29 ♂	2.23	36 ♂	2.35	"	Lysine dihydrochloride (0.75 <i>l</i> (+))

l(+)-lysine was employed. These observations are similar to those which have previously been made on the rat (5).

Data on food consumption in the three studies are given in Table II. In computing averages for the animals in the tryptophane series only the 40 day experimental period was considered. As

was true for the rat, the average daily food consumption was almost invariably higher for the animals which showed the more rapid growth. On the whole the mice seemed to scatter food to a greater extent than we have noted with rats. To avoid this half ounce, opal glass ointment jars (low form) were used as food containers. Through the lid of each was punched a hole $5/8$ inch in diameter. For use by mice which scattered food badly these were placed in 100 cc. beakers, into which they fit nicely. Corrections for scattered food were always carefully made.

With respect to the utilization of the isomeric modifications of tryptophane and histidine for growth one may conclude that the differences between the albino mouse and the albino rat are quantitative, rather than qualitative, in character. To judge by comparison with observations on the rat (7), the mouse probably does not invert the unnatural isomers of these amino acids rapidly enough to meet the requirements of growth. The underlying cause may be slower rate of inversion, more intensive metabolic demands, or both. Apparently unnatural lysine does not undergo appreciable inversion. These studies are being extended to compare similarly the optical isomers of other amino acids and to determine whether the amino acids essential to the mouse are the same as those required by the rat.

SUMMARY

The optical isomers of natural tryptophane, histidine, and lysine have each been prepared and fed to mice in diets deficient only in the corresponding natural form, and under conditions analogous to those previously employed in similar studies on rats. The growth promoted by the unnatural isomers of tryptophane and histidine is slower than that induced by the modifications which occur naturally in proteins. The response is quantitatively, but not qualitatively, at variance with that observed in rats. Unnatural lysine, in the mouse as in the rat, fails to promote growth when added to diets deficient in natural lysine.

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STUDIES IN PROTEIN METABOLISM

VIII. THE ACTIVITY OF THE α -AMINO GROUP OF HISTIDINE IN ANIMALS*

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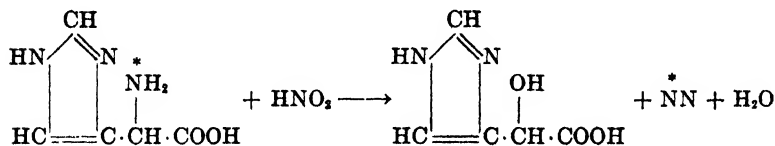
In experiments in which animals were given ammonia (N^{16}) (1), or tyrosine (N^{15}) (2), there were isolated from the proteins several amino acids which contained significant amounts of isotopic nitrogen. The diamino acids, lysine and ornithine from arginine, contained none. The amino acids which contained nitrogen so marked were histidine, glycine, glutamic acid, aspartic acid, and proline. All but the first of these are monoamino acids, in which the isotope must have been located in the α -amino group and must have been derived in the one experiment from ammonia and in the other from tyrosine. As the nitrogen atom in amino acids is stably bound (3), the transfer of nitrogen into an amino acid from another nitrogenous compound cannot occur by mere physical exchange. It must be due to chemical reactions involving both substances, the one of which acted as nitrogen donor (ammonia or tyrosine) and the other as acceptor (other amino acid).

This paper is concerned with this biochemical reaction only in so far as it leads to the introduction of new nitrogen into histidine. Two different reactions might be responsible for the biological uptake of new nitrogen into amino acids: (a) the formation of the amino acid from other substances, involving synthesis of the carbon skeleton, and (b) deamination of the amino acid, followed by amination of the reaction product.

Histidine contains 3 nitrogen atoms, 1 in the α -amino group,

* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

and 2 in the imidazole ring. It was hoped that if the isotope could be located at 1 of the 3 nitrogen atoms in the compound isolated from the animals, insight could be obtained into the chemical reaction to which the amino acid had been subjected. A small amount of isotopic histidine from the proteins of rats which had been given isotopic ammonia (1) was treated with nitrite (4), and the resulting imidazolelactic acid subjected to isotope analysis (5). As it was found to contain normal nitrogen, all of the isotope must have been present in the nitrogen of the



α -amino group.¹ As the total histidine contained 0.013 atom per cent N^{15} excess, the α -nitrogen atom possessed 3 times this concentration; *i.e.*, 0.039 per cent.

The process which led to the introduction of the marked nitrogen into histidine had therefore involved only the α -carbon atom, and consisted of successive deamination and amination. From the experiment in which isotopic tyrosine was administered there was not enough histidine available for the degradation, but it is highly probable that in this case also the new nitrogen was in the α -amino group alone.

This result is in agreement with the known fact that histidine

¹ The marked nitrogen atom, containing more N^{15} than normal, is designated in the formula by the asterisk. We propose to use this sign for the designation of all such atoms marked by an increase of isotope content. We suggest its use also for deuterium-containing compounds: alanine with

marked hydrogen at the α position will be written $\text{CH}_3\text{C}-\text{COOH}$; the use

$$\begin{array}{c} \text{NH}_2 \\ | \\ \text{CH}_3\text{C}-\text{COOH} \\ | \\ \text{H}^* \end{array}$$

of the letter D, as in $\text{CH}_3\text{C}-\text{COOH}$, will be reserved for compounds in

$$\begin{array}{c} \text{NH}_2 \\ | \\ \text{CH}_3\text{C}-\text{COOH} \\ | \\ \text{D} \end{array}$$

which the hydrogen atom in question consists entirely of deuterium.

is an indispensable amino acid, the carbon skeleton of which the animal organism is unable to synthesize in appreciable amounts. However, it is known that when histidine is absent from the diet, the animal organism has the ability to introduce the α -amino group into imidazolelactic or imidazolepyruvic acid, as both compounds may replace histidine in the diet of growing rats (6). Furthermore, it has been found (7) that under the same conditions the unnatural $d(-)$ -histidine can be converted into $l(+)$ -histidine. This conversion must have involved deamination of the unnatural compound followed by amination of the resulting imidazolepyruvic acid.

The new results with marked nitrogen are in agreement with earlier findings in which animals on ordinary stock diets were given heavy water to drink (8). A number of amino acids, including histidine, isolated from the animals contained stably (carbon) bound deuterium. As only chemical reactions can be held responsible for the introduction of the stably bound hydrogen isotope, it was suggested that histidine (and some of the other indispensable amino acids) had been successively deaminized and aminized. According to the generally accepted view on biological synthesis and deamination of amino acids, the intermediates in this process must be keto acids and the reaction involves the hydrogenation of a double bond. If this is carried out in a medium of heavy water, as was the case in the animals given heavy water to drink, the hydrogenation must result in the introduction of at least 1 stably bound deuterium atom; namely, at the α -carbon atom.²

The uptake into histidine of marked hydrogen from the body

² The histidine isolated from these animals contained an amount of deuterium corresponding to more than the uptake of 1 hydrogen atom from the body fluids (1.6 and 1.3 atoms respectively, see (8) Table I). This finding is not in disagreement with the reactions formulated above. Through enolization of the keto acids, more than 1 hydrogen atom of the medium may be introduced. The same reasoning applies to the enimino acid

$$\begin{array}{c} \text{NH}_2 \\ | \\ \text{R}-\text{C}-\text{C}-\text{COOH} \\ | \\ \text{H} \end{array}$$

discussed as intermediate by von Euler and collaborators (9).

fluids and of marked nitrogen from other nitrogenous compounds observed in four different biological experiments must, on the basis of the new findings, be explained by the same biological reaction; namely, the successive deamination of the α -amino group followed by amination of the resulting imidazolepyruvic acid. It is noteworthy that this reaction had been observed on histidine isolated from the bodies of normal animals kept on ordinary stock diets. The process of successive deamination and amination of histidine is obviously a normal metabolic event.

EXPERIMENTAL

To 200 mg. of histidine monohydrochloride, obtained from the bodies of rats given ammonium citrate (N^{15}) (1), dissolved in 5 cc. of water, 170 mg. of freshly prepared silver nitrite were

TABLE I
N¹⁵ Content in Histidine and Imidazolelactic Acid

Preparation No.	Compound	Prepared from	N ¹⁵ content
α 140	Histidine HCl	Casein	atom per cent excess 0.001
α 24	" "	Rats given ammonia N ¹⁵	0.013
α 139	Imidazolelactic acid	Preparation α 140	0.002
α 141	" "	" α 24	0.001

added, and the mixture was shaken for 20 hours in a test-tube closed by a capillary. The precipitate was filtered off and silver was removed from the solution with H_2S . The crystals formed in the concentrated solution were twice recrystallized from a small quantity of water. 56 mg. of material were obtained, melting at 204.5° . N (Kjeldahl) found 18.1 per cent, calculated 18.2 per cent.

The determinations of nitrogen isotope were carried out according to the method described in a previous publication (5). The histidine was reported to contain 0.012 atom per cent N^{15} (1). This was the mean of two independent series of analyses. We have again analyzed this preparation on the same days as when samples of the degradation product, imidazolelactic acid, were

investigated. The new value 0.014 per cent is in good agreement with the earlier results. The mean of all these determinations is 0.013 atom per cent N^{15} . On the same days there were also analyzed samples of natural histidine (three analyses) and ordinary imidazolelactic acid (two analyses). The values given in Table I are the mean values. Both preparations of imidazolelactic acid as well as the histidine from casein show the normal abundance of N^{15} within the limits of error.

SUMMARY

1. Isotopic histidine (N^{15}) obtained from the carcass protein of rats which had been given ammonia N^{15} was converted into imidazolelactic acid. As this compound contained normal nitrogen, the isotope in the histidine was only in the α -amino group.

2. This finding taken together with the results of experiments in which animals were given tyrosine (N^{15}) or heavy water indicates that histidine, even in normal adult animals kept in energy and nitrogen equilibrium, is subject to a continuous process of successive deamination and amination involving only the α -amino group.

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THE PROBABLE IDENTITY OF α - AND β -LINOLEIC ACIDS*

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Preliminary to a study of the glycerides of cottonseed oil, it was found necessary to prepare pure linoleic acid for the synthesis of certain glycerides. The only method available for the preparation of this acid in the quantity and degree of purity desired involved the bromination of free acids of an oil rich in linoleic acid and subsequent reduction of the crystalline tetrabromostearic acid with zinc. However, some doubts have been expressed as to whether linoleic acid thus prepared is identical with the natural acid in the oil, since it has been repeatedly noted that, besides the crystalline bromide, a liquid bromine addition compound is formed. This observation led Bedford (1) to suggest that two isomeric linoleic acids existed. To differentiate the two forms, he designated as α -¹ the crystalline bromide and its regenerated linoleic acid; as β -, the liquid bromide and its regenerated linoleic acid.

Although this suggestion has been given support and credence by some authors, others have held the opinion that only one geometrical configuration of linoleic acid exists. If the latter view is correct, the α - and β -tetrabromides must be regarded as racemic isomers, which upon reduction with zinc should regenerate the same linoleic acid.

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¹ The terms α and β are used throughout this paper merely for convenience and not to imply configurational significance.

That the regenerated linoleic acid from the α -bromide is identical with natural² linoleic acid has been shown by the work of Rollett (2), Birosel (3), Brown and Frankel (4), and others. They have established that the bromination of α - and natural linoleic acid produces the same crystalline tetrabromostearic acid, m. p. 114–115°, in yields approaching but not exceeding 50 per cent of theory. They obtained also, somewhat similar yields of a liquid tetrabromide (β -).

The regenerated linoleic acid from the β -bromide, however, has not been extensively investigated and the few observations reported are not convincing that it is identical with α - and natural linoleic acid. Rollett (2) obtained impure β -linoleic acid (iodine number 159.5; theory 181.4), which upon bromination gave only 26.2 per cent yield of crystalline bromide, m. p. 113–114°. Similarly, Green and Hilditch (5) obtained an impure β -linoleic acid, iodine number 133.1, which upon oxidation with alkaline permanganate gave only 5 per cent of the theoretical amount of mixed sativic acids, whereas the same procedure applied to α -linoleic acid gave 65 per cent. From their work on β -linoleic acid the latter authors concluded: "From the oxidation results, it appears still to contain a certain amount (perhaps 10–12%) of the α -acid; whilst, from its low I. V. and other considerations, we are inclined to suspect that part, at least, of this product has undergone more profound alteration than the mere conversion of cis- into trans-ethenoid linkages."

Analogous to the bromination, the alkaline permanganate oxidation of linoleic acid would be expected to produce two tetrahydroxystearic acids (sativic acids). There appears to be at least some evidence that such is the case. However, contradictory information exists concerning these oxidation products. Hazura (6) reported a series of fractions of indefinite melting points varying from 154° to 173° in his attempts to identify the sativic acids. Nicolet and Cox (7) were the first to report two sativic acids, one melting at 153° and the other at 170°, from a single oxidation experiment. Green and Hilditch (5) obtained the values 155° and 173°, whereas Birosel (8) in recent work reported 163° and 174°. All of these authors, with the

² The term "natural" refers to linoleic acid present in the mixed acids of an oil or to linoleic acid obtained by crystallization of such mixed acids

exception of Green and Hilditch, limited their oxidation studies to α - and natural linoleic acid.

From the foregoing, it was considered desirable to attempt the preparation of β -linoleic acid in reasonably pure form and to compare the products of bromination and oxidation of this acid with those obtained by similar treatment of α -linoleic acid.

Accordingly, in the investigation herein reported both α - and β -linoleic acid were prepared. These were obtained in reasonably pure state as judged by the iodine number, neutralization equivalent, and other constants. The products isolated from the bromination and oxidation of β -linoleic acid were found to be identical with those similarly obtained from the α acid. Only two tetrabromides and two sativic acids could be isolated.

The above facts, taken in conjunction with certain theoretical considerations which are discussed later in this paper, seem to indicate that α -, β -, and natural linoleic acids are identical and that two tetrabromostearic acids and two sativic acids may reasonably be expected on the basis of a single geometrical configuration for linoleic acid.

EXPERIMENTAL

Source of Starting Material—1400 gm. of the total mixed acids of cottonseed oil were dissolved in 5 liters of acetone. The solution was allowed to stand overnight at -15° , after which the separated solids were removed by suction filtration. 844 gm. of unsaturated acids, iodine No.³ 150.0, were obtained from the filtrate. This portion was brominated in ether at -10° to 0° according to Rollett's procedure (2). 579 gm. of crystalline tetrabromides, m. p. 114.5 – 115.0° , were obtained. No evidence of any other crystalline or solid bromide was observed.

Preparation of α -Methyl Linoleate—The debromination of the crystalline bromide was accomplished by treatment with zinc and methyl alcohol, as described by Kimura (9). From a 50 gm. portion, 22.6 gm. of water-clear, distilled methyl linoleate were obtained, iodine No. 171.2, theory 172.6; saponification equivalent 294.5; $n_D^{25} = 1.4592$. The yield is 92.3 per cent of theory.

Bromination of α -Linoleic Acid—33 gm. of linoleic acid obtained

* The Wijs method (1 hour) was used for all iodine numbers determined.

by saponification of 35 gm. of α -methyl ester were brominated in 330 cc. of anhydrous ether at -10 to 0° . A three neck, 1 liter balloon flask equipped with an efficient mechanical stirrer and a dropping funnel was used. After the removal of the slight excess of bromine with a few drops of amylene, the first crop of crystals was filtered off. The filtrate was concentrated to about 150 cc. and a second crop taken. These solid bromides were recrystallized from a mixture of ether and petroleum ether (1:2). 32.1 gm. of α -tetrabromostearic acid, m. p. 115.0 – 115.3° , were obtained. Yield 45.4 per cent. A mixed melting point with the first preparation, m. p. 114.5 – 115.0° , showed no depression.

The ether was evaporated from the filtrate of the second crop of crystals. To this residue 200 cc. of petroleum ether were added and the solution cooled at -15° overnight. The semisolid material which separated was removed by filtration. It weighed 3.2 gm. and appeared to be a mixture. The petroleum ether of the filtrate was removed by distillation; the final traces under reduced pressure. The slightly straw-colored oily residue of β -tetrabromostearic acid weighed 34.0 gm. Yield 48.1 per cent of theory. Attempts to crystallize it from petroleum ether resulted in a tacky, wax-like mass.

Preparation of β -Methyl Linoleate—33 gm. of the β -tetrabromostearic acid were debrominated in the same manner as described. The product obtained as the methyl ester was fractionated by distillation under reduced pressure four times, since it became apparent that higher boiling material was present. A water-clear product comparable in boiling point to α -methyl linoleate was obtained. Fractions 1 and 2 (11.07 gm.) represent a yield of 70.1 per cent of theory. The analyses of the fractions are given in Table I. The presence of higher boiling material was not apparent in the distillation of the α -methyl linoleate previously described. It should be noted, however, that any side products formed on bromination would likely give rise to contaminants in the β -linoleic acid. Rollett (2) also called attention to the presence of higher boiling material but did not effect a good separation. Fractions 2 and 4 had maleic anhydride (Diene) values of 4.6 and 23.2, respectively, which, in view of their higher saponification equivalent, may indicate that to a small extent conjugate

linkages were formed and that subsequent polymerization or cyclization involved the carboxyl group.

It appears from these data that at least the greater portion of the original β -methyl linoleate fraction had chemical constants which were similar to those of α -methyl linoleate and reasonably close to theory.

Bromination of β -Linoleic Acid—2.39 gm. of β -linoleic acid, obtained from Fraction 1 by saponification, were brominated in 25 cc. of anhydrous ether at -10° . 1.88 gm. of crystalline tetrabromostearic acid, m. p. 114.0 – 114.5° , were obtained in the same manner as described. A mixed melting point with α -tetrabromo-

TABLE I
Analysis of Fractions Obtained by Distillation of β -Methyl Linoleate

Fraction No.	Weight*	Iodine No. (Wijs)	Saponification equivalent	Refractive index, 25°
	gm.			
1	7.75	170.9	295.0	1.4591
2	3.32	168.1	297.1	1.4592
3	0.64	154.1	302.9	1.4591
4	1.29	129.3	313.1	1.4592
Residue	1.60	97.3		1.4593

* These weights were corrected for transfer losses and for samples removed for analyses.

stearic acid, m. p. 115.0 – 115.3° , produced no depression. The liquid bromide also formed was not investigated.

The yield of crystalline bromide, 36.7 per cent, is about 10 per cent lower than was obtained on bromination of α -linoleic acid. Although adequate explanation for this is lacking, the yield is large enough to indicate that the greater portion of the β acid must be identical with α -linoleic acid.

Oxidation of α -Linoleic Acid—Fifteen separate alkaline permanganate oxidations of α -linoleic acid were carried out in an attempt to determine conditions by which satisfactory yields of sativic acids could be obtained. Temperatures of reactants were varied between 0° and 30° . Wide ranges of concentration of reactants and time of reaction were employed. Repeated at-

tempts to duplicate the yield of 88 per cent of theory of sativic acids, m. p. 171–172°, reported by Haworth (10), resulted in yields of 50 to 60 per cent, m. p. 154–163°. However, reproducible yields of sativic acids, 61 to 62 per cent of theory, m. p. 154–163°, were obtained by slight modifications of his procedure as follows: 300 cc. of 1 per cent potassium permanganate were added rapidly to a solution of 2.5 gm. of linoleic acid in 300 cc. of 1.5 per cent potassium hydroxide. The temperature of both solutions was 28–29°. The mixture was shaken for 2 minutes, decolorized with sulfur dioxide, and 50 cc. of 36 per cent hydrochloric acid added. The precipitate was filtered by suction and washed with several small portions of water, acetone, and ether.

Separation and Characterization of the Sativic Acids—In order to determine how many individual sativic acids and the approximate proportion of each which were represented in the crude oxidation product, the following general procedure was developed for their separation and characterization. 10 gm., representing a portion of the combined crude oxidation product (m. p. 154–163°) obtained from fifteen oxidations, were first crystallized from 30 per cent acetic acid to remove soluble occluded impurities. The crystalline material was then extracted with boiling acetone (200 cc. per gm. of mixed acids) until the melting point of the unextracted material was 168° or higher. Recrystallization of this residue from 50 to 55 per cent ethanol (200 cc. per gm. of residue) gave 3.28 gm. of a pure sativic acid (A), m. p. 174°, neutralization equivalent 349.3. The residue obtained from the acetone-soluble portion by evaporation of the solvent was crystallized several times from 40 to 50 per cent ethanol (200 cc. per gm. of residue) to give 2.90 gm. of a second pure sativic acid (B), m. p. 163.5°, neutralization equivalent 349.3. Theory for the neutralization equivalent of sativic acid is 348.4.

Since repeated crystallizations and partial extractions of the two acids thus obtained failed to change their melting points significantly, it may safely be assumed that they represent two separate and distinct pure sativic acids. Partial optical crystallographic data⁴ as determined by the microscopical examination of

⁴ All optical and crystallographic examinations were kindly made by Mr. G. K. Keenan of the Food and Drug Administration, United States Department of Agriculture.

these acids further confirmed the fact that they were not identical and also served to characterize them. Sativic acid (A), crystallized from 70 per cent ethanol to facilitate examination, consisted of very thin, colorless, rhomboid plates, forming rosette aggregates. When examined by the immersion method, most of these plates failed to show sharp extinction in parallel polarized light (crossed nicols). Occasionally, however, but very rarely, an elongated plate was found which showed straight extinction and negative elongation. In convergent polarized light (crossed nicols) sections perpendicular to an optic axis are common, these showing the emergence of an optic axis. On such fragments, the index of refraction by the immersion method was 1.535 and may be considered as the β value. The lowest index of refraction which could be measured and only found after considerable searching was approximately 1.532 (n_α). The maximum value, also not common, was approximately 1.555 (n_γ). It is therefore apparent that the double refraction is extremely strong ($n_\gamma - n_\alpha = 0.023$, approximately). A sample of sativic acid (B), crystallized from 70 per cent ethanol, had a different appearance from sativic acid (A) when examined microscopically. The former consisted of rosette aggregates of very thin micaceous plates, which did not show any definite geometrical form like the latter. When examined under the microscope in ordinary light, the material representing the (B) acid was colorless, breaking up into thin papery fragments which showed a tendency to remain suspended on edge in the immersion liquid, giving the impression of needles. No brilliant polarization colors were shown with crossed nicols, mostly first order white being in evidence. The lowest index of refraction that was measured on plates that could be rolled over from their edgewise position was 1.505, which was considerably lower than the lowest value obtained for the (A) acid. This was the only index value that could be determined with certainty. No interference figures were observed in convergent polarized light (crossed nicols).

In addition to the two sativic acids which were obtained as previously described, further small quantities of crystalline material were obtained by concentrating the alcoholic mother liquors to small volume and cooling. 0.82 gm. (A_1), m. p. 157–161°, neutralization equivalent 347.8, was obtained from (A) and 1.58

gm. (B_1), m. p. $153-155^\circ$, neutralization equivalent 349.5, was obtained from (B). In view of the fact that others have reported the presence of a pure sativic acid melting at 153° or 155° , it seemed desirable to ascertain whether these fractions consisted merely of mixtures of (A) and (B) or contained a third sativic acid. Accordingly, a series of mixtures of sativic acids was prepared containing weighed amounts of pure (A) and (B). The melting points of these mixtures were determined in accordance with the Rast (11) technique. The data are shown graphically in Fig. 1.

The temperatures used correspond, as in the Rast method, to

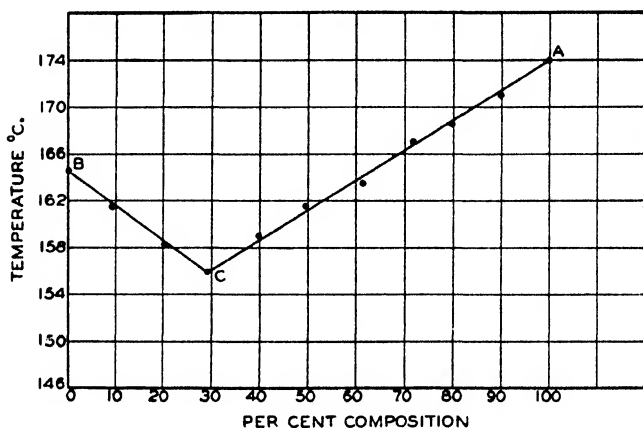


FIG. 1. Melting points of known mixtures of sativic acids

the readings at which the last trace of solid melted. It will be noted from this graph that a melting point of 156° , which represents a eutectic composition of approximately 30 per cent (A) and 70 per cent (B), corresponds very closely to the values reported for a pure sativic acid by some authors. From the portion of the curve C-A, the composition of fraction (A_1) corresponds to approximately a 1:1 mixture. Fraction (B_1) corresponds closely to the eutectic mixture. Calculation from the graph of the composition of these two fractions, when losses during the separation procedure are allowed for, makes it possible to estimate that the composition of the original mixed sativic acids corresponds to about equal amounts of (A) and (B).

Oxidation of β -Linoleic Acid—3.62 gm. of β -linoleic acid (iodine No. 170.9) were oxidized in the manner described. After several crystallizations from 30 per cent acetic acid, 1.75 gm. of mixed sativic acids, m. p. 152.5–160.5°, were obtained. This material when separated as described above gave 0.51 gm., m. p. 173.5–174°, and 0.32 gm., m. p. 162.5–163.0°. Each of these specimens, when mixed with the corresponding sativic acid obtained from α -linoleic acid, gave no depression in melting point.

Also 0.41 gm. of mixed sativic acids, m. p. 153–157°, were recovered from the alcoholic mother liquors. This melting point corresponds closely to that of the eutectic composition (Fig. 1). Similar calculation as employed above would indicate that the two acids were formed in equal amounts. The yield of the two sativic acids, however, was only 39 per cent as compared to 62 per cent obtained on oxidation of α -linoleic acid.

From the study of the melting points of known mixtures of the two acids, it appears that the sativic acid melting at 153° or 155°, which has been reported by others as a pure compound, was a mixture of sativic acids (A) and (B) of nearly the eutectic composition.

DISCUSSION⁵

From the results reported in this paper and from some observations of others, it has been shown that essentially only two tetrabromostearic acids are obtained on bromination of α -, β -, or natural linoleic acid; likewise only two sativic acids are obtained on alkaline permanganate oxidation of each linoleic acid. These facts make it appear that the α -, β -, and natural linoleic acids obtained from the more common seed oils are identical.

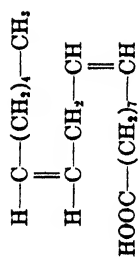
Further consideration would also indicate that two bromination and two oxidation products may reasonably be expected on the postulation of but one geometrical configuration for linoleic

⁵ Since the completion of the above investigation, a very recent paper by McCutcheon (12) has been called to our attention. Although the general conclusions regarding the probable identity of α - and β -linoleic acids are essentially the same as those of the present authors, the explanation of the probable mechanism by which the bromination products may be formed is somewhat different. The present report, besides containing additional information concerning the preparation and bromination of α - and β -linoleic acids also deals with a study of the alkaline permanganate oxidation products of these acids.

acid. It is well known that the action of bromine on certain geometrical isomers, such as maleic-fumaric, cinnamic-allocinnamic, and oleic-elaidic acids, produces essentially either *cis*- or *trans*-addition compounds, but not both in the same reaction. This behavior is known as *cis*- or *trans*-addition. The action of bromine on oleic-elaidic acids apparently gives only *cis*-addition, from interpretation of the work of Holde and Gorgas (13). Oxidation with alkaline permanganate, as a general rule, gives only *cis*-addition of hydroxyl groups to the double bond. If exclusively only one type of addition takes place to compounds containing one unsymmetrically substituted double bond, then only one of the two possible addition products (racemates) is formed. Linoleic acid contains two such double bonds. If it is assumed that only one type of addition has taken place to each of the ethenoid linkages of a single geometrical form of linoleic acid, only two racemic addition products are to be expected. The accompanying schematic representation may serve to picture the addition products that may be formed by *cis*-addition to the *cis*-*cis* isomer of linoleic acid.

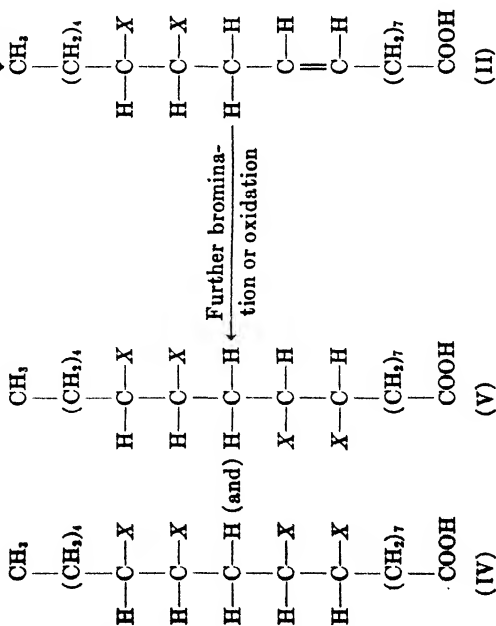
If it is considered that the $\Delta^{12, 13}$ position of *cis*-*cis*-linoleic acid (I) is first attacked, then the isomers (II) and (III) would be formed as intermediates, which on further bromination or oxidation would give rise to the end-products (IV), (V) and (VI), (VII), respectively. It will be noted that the end-products (IV) and (VII) are optical isomers and together would constitute a racemate. Likewise, products (V) and (VI) would represent a second racemate.

For the purpose of illustration, the *cis*-*cis* configuration for linoleic acid, which is considered the more likely form from analogy to oleic acid, has been used to represent the addition products. It is interesting to note, however, that the same reasoning applied to each of the other three possible geometrical isomers of linoleic acid, the *cis*-*trans*, *trans*-*cis*, and *trans*-*trans* forms, would lead to two different racemates. Thus, if the four geometrical isomers were present, the eight theoretically possible racemic addition products would be formed. The experimental data show that only two are obtained; hence the postulation of one geometrical configuration for linoleic acid appears justified. It may be further pointed out that if only *trans*-addition instead



(I)

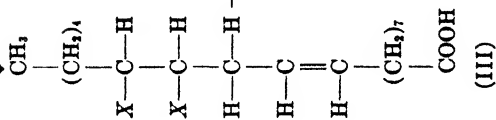
Probable
first step in
bromination
or oxidation



(IV)

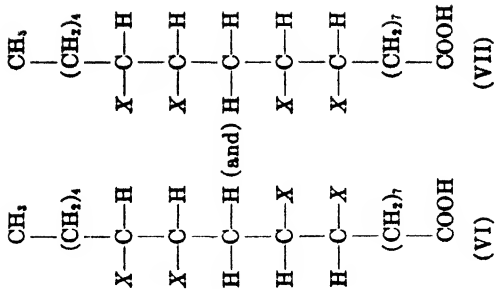
(V)

Further bromina-
tion or oxidation



(III)

Further bromina-
tion or oxidation



(VI)

(VII)

of cis-addition were assumed to take place, the same reasoning would hold; forms (IV), (V), (VI), and (VII) would be produced by trans-addition to trans-trans-linoleic acid.

SUMMARY

1. α - and β -linoleic acids of similar chemical constants were prepared; their bromination and alkaline permanganate oxidation products were studied.

2. Only two tetrabromostearic acids and two sativic acids were obtained. With the exception of the liquid tetrabromide, these products were separated in pure form and characterized.

3. It is believed that α -, β -, and natural linoleic acids are identical and have but one geometrical configuration.

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THE METABOLISM OF CHLORINATED NAPHTHALENES

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Chlorination products of naphthalene and related compounds are of considerable interest because of their wide industrial use and possible toxic properties. The toxicity of a number of these compounds has been worked out in detail (1), and the pathological changes which result from their action have been studied (2). The injurious effects are manifested by degenerative changes in the liver (2, 3) which are somewhat different from the changes produced by chloroform and carbon tetrachloride (2). Nevertheless, small (sublethal) doses of carbon tetrachloride produce a high percentage of fatalities when administered to animals which have been exposed to certain chloronaphthalenes and chlorinated diphenyls (1). The toxicity of the chloronaphthalenes and allied substances tends to increase as the number of chlorine atoms in the molecule is increased. A mixture of trichloronaphthalenes is relatively innocuous, whereas a mixture of penta- and hexachloronaphthalenes produces considerable liver damage when given in small amounts (1).

Apparently the mode of action of these compounds depends in some way on the presence of chlorine in the molecule, and the problem of their toxicity is closely related to the general problem of the toxicity of organic chlorine. It was interesting and important, therefore, to determine as far as possible the fate of these compounds in metabolism. It is the purpose of this paper to report the results of experiments which indicate the main pathways in the metabolism of one of the compounds previously studied (Compound D of Bennett, Drinker, and Warren (2)).

EXPERIMENTAL

Material for Experiment—All experiments reported here were carried out on a mixture of polychlorinated naphthalenes which from the chlorine content appears to consist largely of penta- and hexachloro compounds. This material, used commercially for electrical insulation, has not as yet been resolved into its components. It contains 62.6 per cent chlorine. It is insoluble in water, ethanol, and acetone; very soluble (30 to 90 per cent) in xylene, benzene, and carbon tetrachloride; and moderately soluble (2 to 4 per cent) in esters, ethyl ether, oleic and palmitic acids, and olive oil. The flow point is at 121° to 125°, and the low vapor pressure is 0.2 mm. at 60° and 0.8 mm. at 130°.

For convenience the material will be referred to hereafter as Compound D.

Absorption—The compound, dissolved in olive oil, was given by stomach tube to albino rats (150 to 200 gm. of body weight) in doses varying from 1 to 15 mg. per day. The feces were collected for 3 day periods, and were dried at 80° for 4 days.¹ The material then was pulverized and extracted with benzene in a continuous extractor. After the benzene was distilled off, the residue was transferred to a Carius tube with the aid of a little ether. The ether was removed by vacuum distillation; silver nitrate and nitric acid were added; the tube was sealed and heated for 2 hours at 300°. The silver chloride was estimated gravimetrically by the method of Pregl (4). The chloride found in the extracts varied from 0.1 to 0.4 mg. of chlorine compared with 0.4 mg. of benzene-extractable chlorine found in the excreta of a control animal over a comparable period of time. To each of two control samples, 10.0 mg. portions of finely ground chloronaphthalene were added before drying. The chloride recovered was equivalent to 84 and 85 per cent of the organic chlorine added.

Apparently, therefore, the rat can absorb chloronaphthalene given by mouth nearly quantitatively in amounts up to 15 mg. per day. When larger amounts were given (up to 40 mg. per day) the feces became semiliquid, and impossible to collect

¹ Finely ground Compound D lost less than 0.1 per cent of its weight when heated in the oven under the same conditions.

separately, so no quantitative experiments were undertaken. However, qualitative tests indicated the presence of organically bound chlorine.

Toxicity—Doses of 5 to 10 mg. of Compound D per day were tolerated for a period of 5 weeks, the longest period studied. Doses of 15 mg. per day or greater uniformly killed the animals in 3 weeks or less.

Deposition in Tissues—The observation of Drinker, Warren, and Bennett (1), that liver degeneration in rats which had been exposed to chloronaphthalene was still demonstrable microscopically 2 months after removal from exposure, suggested that deposition of the material in the liver might take place during the period of exposure, followed by a slow liberation of the active toxic material. Consequently, liver tissue and later other organs and tissues from exposed animals were analyzed for organically bound chlorine. The tissues and organs were dried in the oven at 80° for 1 to 4 days. The dry material then was ground in a mortar and extracted with benzene. The benzene was removed by distillation and the residue was analyzed for chlorine by the method previously described, or in later experiments the chlorine was liberated by the procedure of Rauscher (5), and estimated either gravimetrically, as above, or volumetrically ((6) pp. 177–179).

Liver—A total of fifteen livers from animals which had died from exposure to Compound D was analyzed. In one liver from an animal fed 15 mg. per day, 0.5 mg. of benzene-soluble chlorine was found; in no other sample was more than 0.3 mg. of chlorine found. Parallel determinations on normal livers gave similar results; a trace (0.1 to 0.3 mg. of chlorine) usually was found in the benzene extract.

Lungs—The lungs from two exposed animals gave only traces of benzene-soluble chlorine.

Skin—Two analyses of skin samples (about 18 sq. cm.) failed to reveal the presence in this tissue of any significant amount of organic chlorine.

Kidney—One analysis of the kidneys from three rats gave a total of 2.7 mg. of chlorine.

The results show that no significant amounts of the chloro-

naphthalene are stored in the tissues examined, and indicate that the compounds must be either metabolized or excreted with considerable rapidity.

Detoxication Products—Naphthalene (7, 8), monobromonaphthalene (9), naphthalene monochloride (9), and bromobenzene (10) when fed to dogs are metabolized in part at least to mercapturic acids. Consequently, a series of experiments on a dog was undertaken to determine whether chloronaphthalenes would be metabolized in a similar manner.

A female dog weighing 15.4 kilos was placed in a metabolism cage and given a diet consisting of casein 240 gm., sucrose 440 gm., together with brewers' yeast 88 gm., peanut oil 100 gm., cod liver

TABLE I

Urinary Sulfur Analyses Following Chloronaphthalene Feeding

The values are given in mg. of sulfur, phenol, and chlorine respectively.

Day	Sulfur analyses			Phenol		Chloride	
	Inorganic SO ₄	Neutral S	Ethereal SO ₄	Free	Conjugated	Inorganic	Total
10*	151	33	6	143	74	633	
11	182	19	24	166	90	932	935
12	128	22	19	116	36	766	760
13	142	22	5	146	89	672	

* 1 gm. of Compound D was given immediately after the collection of this sample was completed.

oil 100 gm., salt mixture (Karr (11)) 11.2 gm. plus 21 gm. of tricalcium phosphate per kilo of diet, and a small amount of meat extract for flavoring. The intake was stabilized at 150 gm. per day with slight loss of body weight. 24 hour urine specimens were collected by catheterization. Chlorides were estimated gravimetrically before and after evaporation and reduction of organic material by the method of Rauscher (5). Total sulfur, and inorganic and ethereal sulfates were estimated by the methods of Fiske (12), free and conjugated phenols by Folin's procedures ((6) pp. 217-219). The data of one such experiment are presented in Table I.

Administration of 1 gm. of Compound D to the dog produced a significant increase in the ethereal sulfate output from a basal

level of 5 or 6 mg. of sulfur to 24 mg. the 1st day and 19 mg. the 2nd day. However, there was no significant change in the neutral sulfur fraction, nor was there any marked change in the urinary excretion of glucuronic acid when measured by the method of Salt² (13).

The chloride analyses gave results of considerable interest. No organic chlorine was found in the urine. However, there was a significant increase in the chloride output above the basal level for the 2 days following administration of the chloronaphthalene.

TABLE II
Excretion of Urinary Chloride during Chloronaphthalene Feeding

Day	Group I		Group II	
	Average urinary chloride	Body weight	Average urinary chloride	Body weight
	40 mg. Compound D daily		Basal diet	
	mg.	gm.	mg.	gm.
1	4.7	172	3.3	169
2	7.3		1.9	
3	6.7	162	4.2	168
4	7.2		1.6	
5	8.2	149	2.0	174
6	7.7		3.8	
Average.....	7.0		2.8	
	Basal diet		Fasting	
7	2.0	135	6.3	159
8	1.4	125	4.6	148
9	1.1	120	1.6	143

The "extra" chloride amounted to 402 mg., a little over 60 per cent of the chlorine fed. This finding suggested the possibility that one of the early steps in metabolism of the chlorinated naphthalene might be the liberation of the chloride. This possibility was tested in a series of three experiments on albino rats, the results of one of which are given in Table II.

² There is some evidence (8) to indicate that the mercapturic acid derivatives of hydrocarbons are excreted as unstable conjugation products of glucuronic acid.

The rats were given a diet of lactic acid-casein, peanut oil, and corn-starch, with cod liver oil and yeast, and Osborne and Mendel's (14) salt mixture modified by substituting lactic acid for the hydrochloric acid. After a few days the chloride excretion became stabilized at a relatively low level. Three animals then were given daily 40 mg. of Compound D dissolved in olive oil by stomach tube and the three controls were given an equal amount of olive oil. The excretion of chloride rose immediately in the chloronaphthalene-fed animals and remained at a high level as long as the material was given. The animals refused to eat after the first few days and lost considerable body weight, which suggested that part of the "extra" chloride excreted might originate in the tissues. However, when chloronaphthalene feeding was stopped, the chloride excretion promptly fell even though the animals continued to lose weight. Moreover, when similar losses in body weight were induced in the control animals by fasting, only small amounts of "extra" chloride were excreted.

DISCUSSION

The metabolic dehalogenation of halogeno-aryl compounds apparently has not been observed previously. The observation that it does occur in the metabolism of polychloronaphthalenes suggests the possibility that the process may take place more generally and previously may have escaped recognition. If this is proved true, the toxic properties of such compounds might be related to the process of intracellular liberation of halogen.

SUMMARY

The metabolism of a mixture of polychloronaphthalenes was studied. The substance was found to be absorbed completely when given in olive oil solution to albino rats in doses up to 15 mg. per day. No significant storage of the material could be detected in lung, liver, skin, or kidney, nor was any significant amount excreted in the urine. Both the rat and dog apparently were able to remove and excrete the chloride promptly. A rise in the urinary ethereal sulfate fraction, but no significant change in the neutral sulfur excretion, was noted following chloronaphthalene feeding in the dog.

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A STUDY OF BLOOD AND BONE PHOSPHATASE IN CHICK PEROSIS*

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Perosis in chicks, a disease characterized by bone deformities, is associated with a high intake of inorganic phosphate, calcium phosphate, or other calcium salts, and a low intake of manganese (1-6). It was thought possible that this type of diet might have some influence on the phosphatase activity of the bones and blood. In 1931 Hall and King (7) studied the bone phosphatase activity of perotic chickens, using the method of Kay (8) for the phosphatase determination, but could find no change. They concluded that the deformity was not due to an abnormal composition or structure of the bone. Gallup and Norris (9) have found that the bones of chicks suffering from perosis are perceptibly shorter and thicker than normal and that the bone development is affected. In our work we have also observed a malformation of the leg bones and this led us to believe that there might be some disturbance in bone metabolism which would affect the phosphatase activity. In this paper we wish to report the changes we have found in the bone and blood phosphatase activities of perotic and normal chicks.

EXPERIMENTAL

Day-old white Leghorn chicks were divided into groups of five chicks each and placed in suitable cages. The chicks were fed as a basal diet Ration 604, which has been described by Clifcorn and

* A preliminary note on this work appeared in *Science*, **88**, 383 (1938).

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associates (6), for the uniform production of perosis. This ration consists of 48 dextrin, 15 dried beef kidney, 71; crude casein, 14; Salts 2 (10) (without manganese), 5; $\text{Ca}_3(\text{PO}_4)_2$, 3; brewers' yeast, 2; alcoholic extract of rice bran, 5; percomorph oil, 3 drops per chick per week. For the production of normal chicks this ration was supplemented with 50 mg. of manganese per kilo of ration (11). At the end of 6 weeks the chicks were removed from the experiment and blood and bone samples taken for the phosphatase determination.

The samples were prepared as follows: The blood was obtained by bleeding the chicks from the carotid artery directly into oxalated tubes. The tubes were centrifuged and the plasma pipetted off and used immediately for the phosphatase determination.

The bone samples were obtained by removing the tibia as soon as possible after killing the chick, weighing, and grinding in an iron mortar with a little water (12). The sample was transferred and made up to volume in a graduated cylinder, so that the suspension contained 5 per cent of bone in water. The samples were placed in Erlenmeyer flasks, 5 drops of chloroform added as a preservative, and the flasks tightly stoppered and allowed to extract at room temperature for 48 hours. The extract was then filtered through a coarse filter paper and the filtrate used for the phosphatase determination.

The phosphatase activity was determined by the method of King and Armstrong (13), modified as follows for use with the Evelyn photoelectric colorimeter.

Reagents—A. A buffer substrate composed of 10.3 gm. of sodium veronal and 1.09 gm. of disodium phenylphosphate was dissolved in water and made up to 1 liter. This solution was preserved in a well stoppered bottle with a few drops of chloroform and stored in the refrigerator when not in use.

B. Phenol reagent of Folin and Ciocalteu (14). This reagent was diluted 1:3.

C. A 20 per cent solution of sodium carbonate, Na_2CO_3 .

D. A phenol solution containing exactly 0.1 mg. per cc. (14).

E. A standard phenol solution and reagent was prepared by mixing 5 cc. of phenol solution (D) with 15 cc. of diluted phenol reagent (B) and diluting to 50 cc. with water. This solution contained 0.01 mg. of phenol per cc.

Determination—10 cc. of buffer substrate (A) are placed in a test-tube and the tube put into a 37.5° water bath for 5 minutes. Add exactly 0.5 cc. of blood plasma or bone extract filtrate to the tube, mix, and allow to remain in the water bath for 30 minutes. At the end of this time add at once 4.5 cc. of diluted Folin's phenol reagent (B), mix, and filter. 2 cc. of filtrate are placed in a 25 cc. volumetric flask, 2.5 cc. of 20 per cent sodium carbonate (C) added, and the mixture made up to volume. Allow to stand for 20 minutes, transfer to a colorimeter tube, and read the color developed, using the Evelyn photoelectric colorimeter with a 6600 Å. filter. With substances of low phosphatase activity more than 2 cc. of filtrate are necessary in order to obtain a readable color.

Zero hour determinations are run on each sample. To 10 cc. of buffer substrate (A) are added 0.5 cc. of enzyme preparation and immediately 4.5 cc. of diluted phenol reagent (B), mixed and filtered. 10 cc. of filtrate are placed in a 25 cc. volumetric flask, 2.5 cc. of 20 per cent sodium carbonate (C) added, and the mixture made up to volume and read as before.

Standards are prepared by taking 5 and 10 cc. of the standard phenol solution and reagent (E) and adding to each 2.5 cc. of 20 per cent sodium carbonate (C) and making up to 25 cc.

A blank is made by taking 3 cc. of diluted phenol reagent (B), adding 2.5 cc. of the 20 per cent sodium carbonate solution (C), and making up to 25 cc. This blank should be colorless.

The mg. of phenol liberated are calculated by subtracting the mg. of phenol of the zero hour tubes from the mg. of phenol of the incubated tubes. This is a measure of the phosphatase activity, 1 *phosphatase unit* being that amount of enzyme which, when allowed to act upon an excess of disodium phenylphosphate at pH 9.0 for 30 minutes at 37.5°, will liberate 1 mg. of phenol.

Results

Blood and bone phosphatase activity was determined on birds receiving Ration 604 as the basal ration, on birds receiving this diet supplemented with manganese, and also on chicks receiving Ration 604 plus 3 mg. of magnesium per week by injection. The results are given in Table I.

From Table I it can be seen that birds suffering from perosis

have lower blood and bone phosphatase activity than normal birds. In order to decide whether the lowering was due to perosis, or that the lowering preceded the onset of perosis, the following experiment was performed. Day-old white Leghorn chicks were divided into two groups of thirty chicks each. One group was fed Ration 604; the second group, the same basal ration supplemented with 50 mg. of manganese per kilo. Chicks were taken off the experiment every 3 days and blood and bone phosphatase activity determined. The results are plotted in Charts I and II.

From the curves it can be seen that the phosphatase activity of both blood and bone is high in day-old chicks. It then falls rapidly and reaches a fairly constant level. Common (15), using a ration consisting of bran, pollards, corn-meal, ground oats, extracted soy bean meal, salt, and oyster shells, found that the

TABLE I

Blood and Bone Phosphatase Activity of Perotic and Non-Perotic Birds

Ration No.	Perosis	No. of birds	Units of blood phosphatase per 100 cc.*	No. of birds	Units of bone phosphatase per gm. green bone*
604	Yes	22	8.3 (2.1-16.5)	38	4.7 (2.3- 7.5)
604 + Mn	No	21	22.6 (19.6-28.0)	39	9.1 (7.1-15.8)
604 + Mg	Yes	6	13.1	6	4.9

* The figures in parentheses represent the limits.

serum phosphatase activity of chicks showed a rapid increase for about 10 to 12 days after hatching and then fell rapidly to a low level at 3 weeks. As our ration contains a high level of calcium phosphate this may account for the rapid decrease in phosphatase activity we have observed.

It is evident from the records shown in Charts I and II that the chicks receiving the added manganese maintained a higher level of phosphatase activity from very shortly after the initiation of the experiment. Thus it is apparent that the lowering of phosphatase activity definitely precedes the appearance of slipped tendon.

The effect of other calcium salts on the phosphatase activity was also studied. These salts replaced the calcium phosphate in Ration 604 at the same percentage level. The data are given in

Table II. Here again the activity was lowered, but not depressed as far as when the calcium phosphate was fed. A possible explanation for this is seen from our work *in vitro*, described later, which shows the inhibitory effect of the calcium and phosphate ions. The effect of the phosphate ion is absent when calcium carbonate or calcium lactate is fed and only the calcium ion exerts

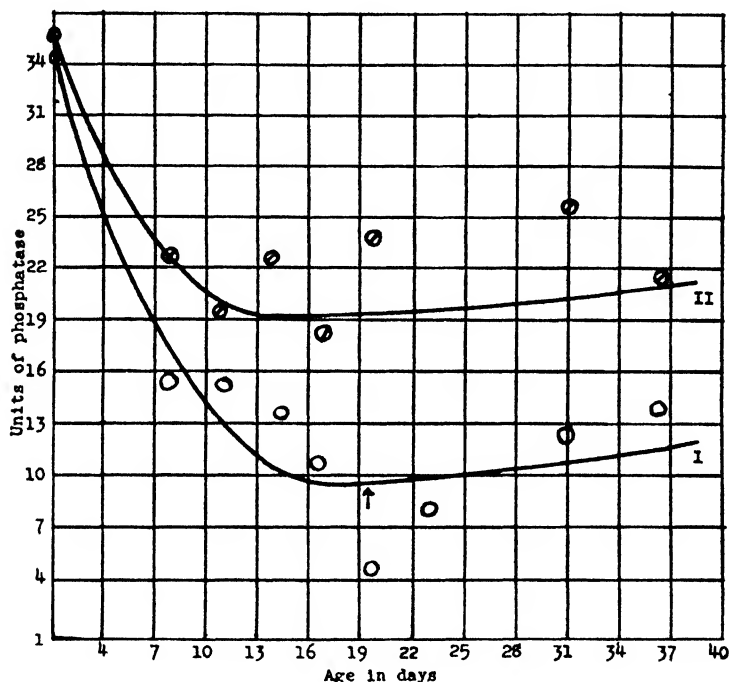


CHART I. ○ represents blood plasma phosphatase activity of perotic chicks; ⊖, blood plasma phosphatase activity of non-perotic chicks. The arrow indicates the first appearance of perosis.

an inhibitory effect. The effect of the calcium lactate on the phosphatase activity is less than that of the calcium carbonate, as the calcium lactate contains only one-third as much calcium.

The blood and bone phosphatase activity of chicks raised on a ration in which the calcium and phosphorus levels were not distorted was determined. The ration used was our grain ration,

No. 351 (16), which consists of yellow corn 58, wheat middlings 25, casein 12, salt 1, CaCO_3 1, $\text{Ca}_3(\text{PO}_4)_2$ 1, yeast 1, cod liver oil 1.

The following figures show that the blood and bone phosphatase activities are higher in these chicks fed ration 351 than in chicks receiving Ration 604: nine chicks, 51.3 units of blood phosphatase per 100 cc.; ten chicks, 9.3 units of bone phosphatase per gm. of green bone.

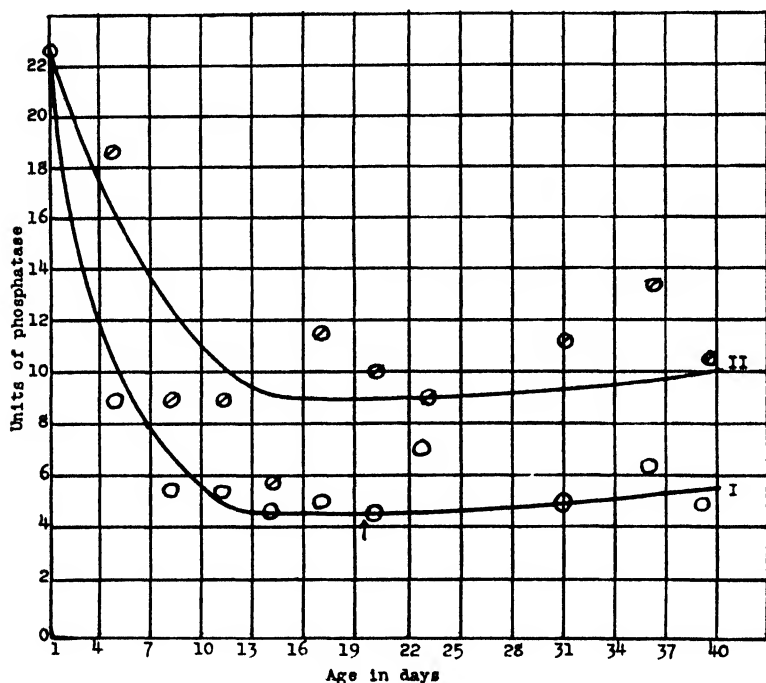


CHART II. ○ represents bone phosphatase activity of perotic chicks; ⊙, bone phosphatase activity of non-perotic chicks. The arrow indicates the first appearance of perosis.

Since the feeding of manganese increased the phosphatase activity, and when large amounts of calcium phosphate were added to the ration there was a lowered activity, it was decided to study the effect of various ions *in vitro*. The data secured are given in Table III.

From these results it may be seen that the enzyme is activated

TABLE II
Effect of Calcium Salts on Phosphatase Activity

Ration No.	Salt used in ration	Peroxis	No. of birds	Units of blood phosphatase per 100 cc.	No. of birds	Units of bone phosphatase per gm. green bone
604	$\text{Ca}_3(\text{PO}_4)_2$ *	Yes	2	13.8	4	5.1
604	CaCO_3	"	4	16.2	5	6.7
604	Calcium lactate	"	5	27.6	5	9.2
604 + Mn	$\text{Ca}_3(\text{PO}_4)_2$	No	5	30.0	5	13.6
604 + "	CaCO_3	"	5	32.6	5	15.8
604 + "	Calcium lactate	"	5	40.8	5	15.1

* All calcium salts were fed at a 3 per cent level.

TABLE III
Effect of Various Ions on Activity of Bone Phosphatase

Ion added	Amount of ion added	Units of bone phosphatase per gm. green bone		Per cent activation or inhibition
		Before	After	
Mn	1 microgram	6.9	7.0	1.5
"	10 micrograms	6.9	7.3	5.8
"	50 "	5.9	8.1	20.4
"	100 "	9.2	12.7	38.1
"	1 mg.	6.4	21.9	242.0
Mg	1.5 "	6.1	9.6	57.0
Ca	0.2 "	12.5	12.5	
"	2 "	12.5	10.8	-13.5
Ca }	0.2 "	12.5	13.7	9.6
Mn }	100 micrograms			
Ca }	2 mg.	12.5	12.6	
Mn }	100 micrograms			
PO_4	0.2 mg. as P	9.2	8.3	-9.8
"	2 " " "	20.8	11.3	-45.5
" }	0.2 "	9.2	9.3	1.1
Mn }	100 micrograms			
PO_4 }	2 mg.	14.6	9.7	-33.3
Mn }	100 micrograms			

definitely by the manganese ion, and to a lesser extent by the magnesium ion. Erdtmann (17) has also found activation of the enzyme by magnesium. Inhibition has been observed with the calcium ion and to a more marked degree with the phosphate ion. This is in accordance with the work of Bauer (18) who found inhibition with the calcium ion, and that of Jacobsen (19) who observed inhibition with the phosphate ion. The addition of manganese overcomes the inhibiting effect of the calcium and phosphate ions. Inhibition has also been found with zinc ions.

The addition of manganese to a bone phosphatase preparation from birds with perosis failed to activate the enzyme to the level of birds without this disorder, and is shown in Table IV. Horii *et al.* (20) have found that manganese salts promote the hydrolysis of certain phosphoric esters by kidney phosphatase.

TABLE IV

Comparison of Additions of Manganese (1 Mg.) to Phosphatase Preparations

Perosis	Units of bone phosphatase per gm. green bone	
	Before	After
Yes	2.1	8.1
No	10.6	35.6

DISCUSSION

We have found the blood plasma and bone phosphatase activity lowered in perosis. In chicks fed a diet high in inorganic phosphate (calcium phosphate) and having a high percentage of slipped tendon, there is some metabolic disturbance of the bone. Since the lowering of the phosphatase activity precedes markedly the appearance of perosis, this seems to be a contributing cause in this condition. Chicks fed the same ration but with a supplement of 50 parts per million of manganese had an approximately normal phosphatase activity as compared to the phosphatase activity of chicks fed a normal grain ration. When a diet high in calcium salts other than the phosphate is fed, there is also a lowering of the phosphatase activity and a high incidence of perosis. The lowering observed is not as great as when high amounts of inorganic phosphate are fed. Here again manganese raises the activity to an apparently normal level. Thus the effect

of the added manganese seems to counteract the effect of the large amount of added phosphate or calcium in the ration. The higher the level of phosphate fed the higher is the amount of manganese required to prevent perosis.

These results agree well with those found in studies *in vitro*. The addition of the phosphate ion to the enzyme markedly decreases its activity. Adding manganese restores the activity, but this activation depends on the amount of manganese ion added as the activator and the amount of phosphate ion added as the inhibitor. This is shown in Table III, and similar results are observed with the calcium ion.

These experiments indicate that the main effect of calcium and phosphate ions is to depress phosphatase activity, while that of manganese ion is to activate the enzyme. However, it was found that when the manganese ion was added to an enzyme preparation obtained from a chick with perosis, the activity did not reach that of an enzyme preparation obtained from a bird without perosis (Table IV). This seems to indicate that the *amount* of enzyme present is less and consequently the manganese added does not bring the activity of an enzyme preparation from a bird with perosis up to that of a non-perotic bird. This would tend to show that the excess calcium and phosphate fed depress the *formation* of the enzyme phosphatase, and that manganese counteracts this depressing effect.

It is well known that magnesium activates the enzyme phosphatase. We have also observed this (Table III) and it was thought that this element might exert a curative effect on this disorder. The chicks were given the basal ration No. 604 and 3 mg. of magnesium per week by injection. This did not prevent perosis (Table I). A possible explanation is that the magnesium does not activate the enzyme phosphatase nearly as effectively as the manganese ion or counteract the depressing effect of the calcium and phosphate ions.

SUMMARY

1. A revised method for the determination of blood and bone phosphatase activity with disodium phenylphosphate as the substrate and applicable for use with the Evelyn photoelectric colorimeter has been presented.

2. The blood and bone phosphatase activity of chicks with perosis is lower than that of non-perotic birds.

3. From *in vitro* experiments manganese has been found to have an activating influence on the enzyme, while calcium and phosphate ions depress the activity.

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PROPERTIES OF THE LACTIC ACID-RACEMIZING ENZYME OF *CLOSTRIDIUM BUTYLICUM**

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(Received for publication, August 5, 1938)

In previous publications (1-3) it was shown that the acetone-butyl alcohol organisms *Clostridium acetobutylicum* and *Clostridium butylicum* have the curious property of converting *d*- or *l*-lactic acid into the inactive *dl* form. Katagiri and Kitahara (4, 5) found that many lactic acid bacteria and *Staphylococcus ureæ* also possess this racemizing ability, but that other bacteria and many yeasts and molds do not have it.

In a preceding paper (3) racemization was attributed to an enzyme which was thought to consist of two components: one found in the cells and heat-stable; the other liberated into the medium and heat-labile. Neither cells nor medium functioned separately, and hence it was assumed that the racemizing system consisted of an enzyme-coenzyme complex. More extended study of the problem has shown that it is not necessary to assume the existence of an extracellular component and an intracellular component. The earlier results may be explained by the distribution of enzyme between cells and medium and by the pH requirements of the enzyme.

The present paper deals with these and other factors influencing the activities of the enzyme. Such a study, it was hoped, would lay the groundwork for elucidation of the function of the enzyme.

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EXPERIMENTAL

Culture and Medium—The organism used in this investigation was *Clostridium butylicum*, No. 21. It was always grown in a 0.5 per cent glucose-0.5 per cent tryptone medium. This is the same organism and the same medium used in the studies reported by Tatum *et al.* (3).

Preparation of Calcium Lactate—Dextrorotatory lactic acid in the form of its calcium salt was used. It was prepared in large quantities by fermenting glucose with a strain of *Lactobacillus delbrückii*, according to the method of Tatum and Peterson (6). Calcium lactate obtained from different fermentations, after several recrystallizations from water, always showed a specific rotation of -6.1° to -6.3° in a 4 per cent solution. Calcium determinations gave practically theoretical results for calcium lactate.

Methods

All of the work so far reported on racemization of active lactic acid has been based on water of crystallization of the zinc lactate as a measure of the change. This method, however, requires much time and is too inaccurate for a quantitative study of the enzyme activity. For this reason a shorter and more accurate method was needed. Such a method, based on the optical rotation of calcium *d*-lactate, was developed.

Rotation of Calcium d-Lactate—Preliminary experiments on the optical properties of calcium *d*-lactate showed that the specific rotation decreases with increasing concentration. However, during racemization experiments the total concentration of calcium lactate remains the same, although the concentration of the *d*-lactate decreases. It was thought that under such conditions the rotation might be closely proportional to the concentration of the dextro acid and hence permit calculation of the *dl* acid present. Mixtures of calcium *d*- and *dl*-lactate in various proportions, but always totaling 4 per cent, were made up and read in a 4 dm. tube in the saccharimeter. From the observed readings the composition of the mixtures was determined and compared with the calculated values. The data are given in Table I and show good agreement between observed and calculated values.

It was thus evident that if the method could be applied to cell

suspensions and cultures the tedious process of extracting the acid, preparing the zinc salts, and determining the water of crystallization could be eliminated.

Treatment of Cultures—The method employed for preparing the cultures for analysis was as follows: Calcium *d*-lactate was added in solution to the culture or fraction to be tested. The amount was usually 2 gm. of the anhydrous salt in 50 cc. of solution. The volume was adjusted as desired, toluene was added, and the flask was tightly stoppered, shaken, and incubated at 37°. After incubation the solution was boiled to remove the toluene, and the pH was adjusted to about 7. After addition of norit to decolorize the solution and remove the cells, and of diatomaceous earth to

TABLE I
Rotation of Calcium Lactate Solutions

Composition of solution		[α] _D	Racemization	
Ca <i>d</i> -lactate	Ca <i>dl</i> -lactate		Calculated	Observed
<i>per cent</i>	<i>per cent</i>	<i>degrees</i>	<i>per cent</i>	<i>per cent</i>
4.00	0.00	-6.13	0.0	0.0
3.52	0.48	-5.30	12.0	13.5
3.00	1.00	-4.44	25.0	27.6
2.00	2.00	-3.03	50.0	50.5
1.08	2.92	-1.73	73.0	71.8
0.48	3.52	-0.54	88.0	91.2
0.00	4.00	0.00	100.0	100.0

aid filtration, the solution was filtered on a small Buchner funnel, and the residue well washed. The filtrate and washings were boiled down and made up to a volume such that the resulting concentration of calcium lactate was 4 per cent. The readings on a 4 dm. tube were taken in a saccharimeter.

With cell suspensions the above treatment always yielded water-clear solutions. Many control experiments in which the enzymes were inactivated before incubation gave complete recovery of the the active calcium lactate. Similar treatments of solutions of active calcium lactate alone gave the same results. Therefore, the procedure does not racemize active lactate nor does it remove lactate from solution.

With the cultures or with the cell-free medium certain difficulties

were encountered. Following the above treatment duplicate solutions often showed differences in rotation as high as 10 per cent. Control experiments showed the same discrepancies. The differences were finally attributed to optically active substances which were removed from the medium in unequal amounts by the above treatment. For the quantitative study of the enzyme system, however, it was possible to dispense with the medium as a source of the enzyme, since as will be shown later, the cells under prescribed conditions are active alone.

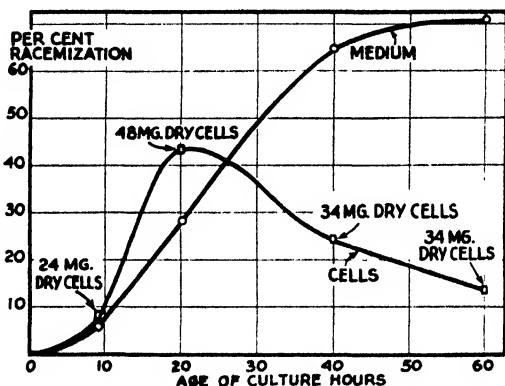


FIG. 1. Distribution of enzyme between cells and medium. The ordinates represent the per cent racemization of 2 gm. of Ca lactate (in 150 cc. of solution) accomplished in 40 hours at 37° and pH 5 by cells or medium from 90 cc. of culture.

Distribution of Enzyme between Medium and Cells

10 liters of culture were sampled at intervals, and the racemizing activity at pH 5 of the cells and of the cell-free medium was determined. The per cent racemization produced by the cells was determined in the usual manner. The medium, however, after incubation with calcium lactate, was acidified and the lactic acid was extracted with ether. The calcium salt of the extracted acid was then made, and its specific rotation determined. The results are shown in Fig. 1. It is seen that the enzyme content of the cells increased up to 20 hours and then decreased as the culture aged. That the initial increase in activity does not parallel in-

crease in cell weight is illustrated by the fact that in from 9 hours to 20 hours the enzyme content increased more than 5-fold, while the cell weight only doubled. The enzyme content of the medium rose rapidly as the culture aged, the enzyme very obviously being liberated into the medium from old cells.

Racemization Studies with Cell Suspensions

In order to study some of the properties of the racemizing enzyme a large quantity of cells was grown. The cells were centrifuged from four different cultures of 14 liters each. The wet cells obtained, about 100 gm., were kept in a frozen condition until used. These cells have been kept over a period of 8 months, during which time they have been repeatedly thawed, and the activity has remained quite constant.

Effect of pH—In preliminary experiments on effect of pH, the solutions were adjusted with hydrochloric acid and calcium hydroxide. It was found, however, that upon readjusting the solutions to pH 7 the rotations of control solutions containing inactivated cells were considerably decreased. However, the use of sulfuric acid and calcium hydroxide for pH adjustments gave theoretical recovery of the rotations of control solutions upon readjustment to pH 7 with the same reagents. In this case the excess calcium and sulfate ions were precipitated upon adjusting and readjusting the solutions, while in the other the presence of excess calcium or chloride ions brought about the decrease in rotations. In all experiments, therefore, sulfuric acid and calcium hydroxide were used as needed for pH control.

Because of the danger of influencing the rotations, as above, no buffers were used. It was found that at pH 5 and below, the lactate had sufficient buffer capacity to hold the pH at the desired value. Above pH 6, where the solutions had very little buffer capacity, the greatest fluctuation was about 1 pH unit. The pH of each solution was determined before and after incubation.

Fig. 2 shows that there is a sharp rise in activity from pH 3 to pH 5 followed by a more gradual decrease in activity as the pH increases. The optimum is clearly at about pH 5. In view of this pronounced effect of pH on the velocity of the racemization reaction, it is possible that the negative results obtained by Kata-

giri and Kitahara (5) with many microorganisms may have been due to unfavorable pH.

From the pH curve it appears that the substrate for the racemizing enzyme may be the lactate ion rather than the undissociated lactic acid. There is appreciable racemization at the higher pH values, where the substrate is present entirely as lactate ion. At the lower pH values, much of the substrate is present as free lactic acid, and consequently the concentration of available substrate is lower. In the acid range, therefore, the pH activity curve represents the effect of pH on the available substrate concentration as well as on the racemization reaction itself.

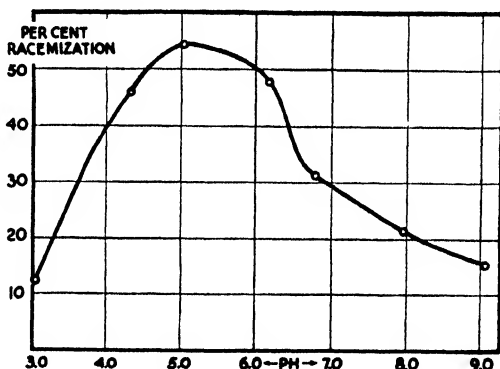


FIG. 2. Effect of pH on enzyme activity. 75 cc. of reaction mixture, containing 2 gm. of calcium lactate and 0.25 gm. of wet cells, were incubated 40 hours at 37°.

Effect of Enzyme Concentration—In Fig. 3 the effect of enzyme concentration on racemization is shown. The solid curve is a theoretical curve for a first order reaction and the experimental points fall upon or close to it.

It may be shown on the following theoretical basis that such a first order reaction curve is to be expected with the racemizing enzyme. Since the product of the reaction is *dl*-lactic acid, the affinity of the enzyme for the *d* and *l* acids must necessarily be identical. Therefore, since the total lactate concentration remains constant, the fraction of the total enzyme present as enzyme-substrate complex remains the same. Of this combined

enzyme, the percentage combined with the *d* acid is always equal to the percentage of *d* acid in the mixture. Thus it follows that a first order reaction is to be expected regardless of the substrate concentration.

Effect of Substrate Concentration—In Fig. 3, the racemization given by a cell suspension is plotted against molar concentration of lactate. It will be noted that the enzyme did not approach maximal activity until a very high substrate concentration was reached (0.5 M). While the data of the figure do not allow an

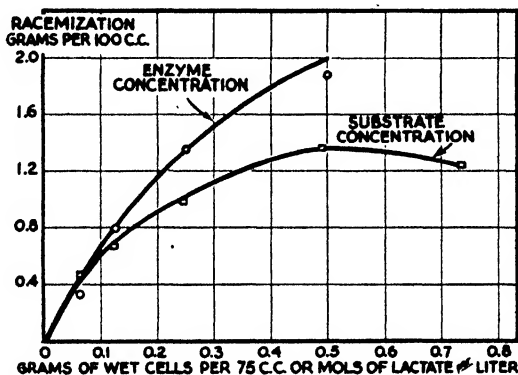


FIG. 3. Effect of variation of enzyme and substrate concentrations on racemization. When the enzyme concentration was varied, the calcium lactate concentration was 2 gm. per 75 cc., and the incubation time was 40 hours. When the substrate concentration was varied, 0.25 gm. of wet cells was present in 75 cc., and the incubation time was 20 hours. In all cases the pH was 5 and the incubation temperature 37°.

exact determination of the Michaelis constant, its value apparently lies in the neighborhood of 0.14, a value much higher than that for most respiratory enzymes.

Effect of Cyanide—As shown in Table II, 0.05 M cyanide completely inhibits racemization. Iron- or copper-containing enzymes are known to be cyanide-sensitive, while most dehydrogenases are not.

Effect of Incubation Temperature—Because of the long incubation periods necessary, thermal inactivation of the enzyme limited measurement of the racemization velocity at high temperatures.

The data of Table III show appreciable inactivation at 45°, and probably some inactivation at 40°. However, the apparent energy of activation of the racemization process is less than that of

TABLE II
Effect of Cyanide on Racemization

100 cc. of reaction mixture, at pH 5, containing 2 gm. of calcium lactate and 0.25 gm. of wet cells, were incubated 46 hours at 37°.

HCN	Racemization	Inhibition
<i>M</i>	<i>per cent</i>	<i>per cent</i>
0.0	36.4	
0.0	34.6	
0.05	0.0	100
0.05	0.0	100
0.005	17.0	53
0.005	15.2	57

TABLE III
Temperature Variation of Racemization Velocity

75 cc. of reaction mixture, at pH 5, contained 2 gm. of calcium lactate and 0.25 gm. of wet cells. The incubation was carried out for 48 hours at the indicated temperatures.

Incubation temperature	$[\alpha]_D$	Velocity constant k^*	Q_{10}^\dagger	μ^\ddagger
$^{\circ}\text{C.}$	<i>degrees</i>			<i>calories</i>
20	-3.81	0.207		
30	-3.03	0.306	1.48	6900
40	-2.38	0.411	1.34	5600
45	-2.82	0.338		
60	-5.20	0.025		

$$* k = \log \frac{[\alpha]_D \text{ of } d\text{-lactate}}{[\alpha]_D \text{ after incubation}}$$

$$\dagger Q_{10} = \text{ratio of velocity constants.}$$

$$\ddagger \mu = \frac{4.58 \log (k_2/k_1) T_1 T_2}{T_2 - T_1}$$

most enzymatic reactions, which usually ranges from 10,000 to 20,000 calories.

Clostridium butylicum Dehydrogenases—While the racemizing activity of washed cells is extremely stable, preliminary experi-

ments showed the cell dehydrogenases to be extremely unstable. The very considerable activity possessed by freshly harvested cells disappeared within a few hours. Table IV gives representative data. It will be noted that even the fresh cells had very little lactic acid dehydrogenase activity, although growing cultures of the organism are able to metabolize lactic acid (3).

TABLE IV
Dehydrogenase Activity of Washed Cells

4 cc. of reaction mixture contained cells from 5 cc. of a 22 hour culture, 1 cc. of 0.02 per cent methylene blue, and 1 cc. of pH 6.4 phosphate buffer. The incubation was carried out at 40° in evacuated Thunberg tubes.

Substrate	Concentration of substrate	Decolorization time	
		Fresh cells	Cells after 3 hrs. at 25°
	<i>M</i>	<i>min.</i>	<i>min.</i>
None.....		> 215	
Glucose.....	0.025	7	18
Isopropyl alcohol.....	0.05	26	69
Pyruvate.....	0.025	22	
Lactate.....	0.075	156	> 220

DISCUSSION

It seems probable that the racemizing enzyme has, in the living cell, a function other than that of racemization.

Any enzyme capable of combination with lactic acid could, under the proper conditions, act as a racemase, provided that the enzyme possessed an equal affinity for the two optical forms of lactic acid and that the enzyme-substrate reaction destroyed the asymmetric structure of the α -carbon atom. This might take place by momentary, reversible conversion of the lactic acid molecule into acrylic acid, pyruvic acid, or methylglyoxal. The extreme instability of the cell dehydrogenases, as well as the cyanide sensitivity of the racemizing enzyme, seems to indicate that the latter is not a lactic acid dehydrogenase. Moreover, bacterial dehydrogenases are usually strongly cell-bound, while the racemizing enzyme is readily secreted into the medium. The substrate affinity of the racemizing enzyme is also much lower than that of a dehydrogenase.

Since growing cultures of *Clostridium butylicum* are able to ferment added lactic acid (3), and will actually produce lactic acid from glucose when grown at high pH values (7), it seems probable that the racemization studied in the present paper is due to an enzyme concerned in the normal lactic acid metabolism of the organism.

SUMMARY

Additional data on the pH requirements and distribution of the racemizing enzyme of *Clostridium butylicum* show that the enzyme does not necessarily consist of two components as was previously concluded.

The enzyme acts most rapidly at pH 5 and at a lactate concentration of 0.5 M. It is completely inhibited by 0.05 M cyanide.

While cells from young cultures are high in enzyme, in old cultures most of the activity is found in the culture medium.

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STUDIES ON THE PRODUCTION OF TAUROCHOLIC ACID IN THE DOG

III. CYSTINE DISULFOXIDE, CYSTEINE SULFINIC ACID, AND CYSTEIC ACID

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(Received for publication, October 24, 1938)

In determining the fate of various sulfur-containing compounds in the human organism, Medes (1) fed cystine disulfoxide ($\text{R}-\text{S}-\text{S}-\text{R}$), cysteine sulfinic acid ($\text{R}-\text{SO}_2\text{H}$), and cysteic acid ($\text{R}-\text{SO}_3\text{H}$). She found that the compound in which the sulfur was most highly oxidized, *i.e.* cysteic acid, was least readily oxidized in the organism as far as could be determined by the recoveries of organic and sulfate sulfur in the urine. The compound whose sulfur was in the lowest state of oxidation, *i.e.* cystine disulfoxide, was oxidized to the greatest extent by the body, judging again by recovery of urinary sulfate. Cysteine sulfinic acid gave intermediate values for oxidized urinary sulfur. Since the series cystine disulfoxide > cysteine sulfinic acid > cysteic acid represented the relative degree of recovery of oxidized sulfur in the urine, Medes believes that this series of compounds does not represent the path of oxidation of cystine to sulfate. She suggests "that the sulphur of the more highly oxidized members of this series may be readily available for some other physiological function, such as taurine formation." To obtain more information concerning these suggestions we have measured the taurocholic acid output of bile fistula dogs after administration of the sulfur-containing compounds.

EXPERIMENTAL

The dog normally forms enough taurine for conjugation with all the cholic acid which is available. Fasting depletes the body of its supply of taurine. Repeated feedings of cholic acid will also

reduce the supply of taurine by increasing its excretion in the bile as taurocholic acid. The two procedures for depletion of the taurine supply were used together in these experiments, as follows: In order to measure the effect of the sulfur-containing substances on the output of taurine, bile fistula dogs were fasted but fed 6.7 milliequivalents of cholic acid daily. When the taurocholic acid production had dropped, a sulfur-containing compound¹ was administered with the cholic acid, and the taurocholic acid content of the bile was determined. The analytical methods used have been described earlier (2). Dog 29 (Table III) was treated somewhat differently than were the other animals, in that it was given 25 gm. of sucrose per day. This was given as a protein sparer because of the relatively long fast to which the dog was subjected.

Results

The data obtained on administration of cystine disulfoxide are presented in Table I. Four attempts were made to feed this substance, but vomiting occurred after three of the four attempts. Results from the successful feeding are those reported for Dog 36. Three subcutaneous injections of the disulfoxide dissolved in a minimum of ammonium hydroxide were made. A necrosis appeared at the site of injection in two cases. Data for those two experiments have been discarded. The data from the one successful subcutaneous injection make up the first experiment on Dog 39. Two further administrations of the disulfoxide were made intravenously. The data for the second experiment with Dog 39 and the experiment with Dog 45 present these results. In all four successful experiments an increased excretion of taurocholic acid followed the administration of cystine disulfoxide. The increases were not so large as those found after administering cystine (2) or its more highly oxidized derivatives (cysteine sulfinic acid and cysteic acid; see below). These results may be complicated by the apparent toxicity of the disulfoxide, although no consistent rise in the nitrogen excretion was observed when the disulfoxide

¹ We wish to acknowledge the suggestion by Dr. Grace Medes that these studies be carried out with cystine disulfoxide and cysteine sulfinic acid, and the kindness of Dr. Medes and others of the Lankenau Hospital Research Institute in Philadelphia in furnishing these two compounds for the experiments herein reported.

was given. The increase of taurocholic acid excretion in the second experiment with Dog 39 is small, but it should be noted that the amount of disulfoxide injected (3.35 milliequivalents) was only half the amount used in other experiments. This smaller injection was given because of the toxicity of cystine disulfoxide.

TABLE I

Urinary Sulfur Partitions and Taurocholic Acid Production Following Administration of Cystine Disulfoxide to Fasting Bile Fistula Dogs

Each animal was fed 2.8 gm. = 6.7 milliequivalents of cholic acid daily.

Dog No.	Day	Weight	Total N	Total S	Sulfate S	Organic S	—SS— S	Taurocholic acid
		kg.	gm.	mg.	mg.	mg.	mg.	mg.
36	1	9.9	3.40	185	153	32	Not run	2353
	2		3.31	158	123	35		2021
	3		3.82	318	282	36		2726*
	4	8.5	3.21	128	95	33		2467
39	1	10.4	4.66	260	123	137	10	1524
	2		2.72	186	93	93	5	712
	3		3.19	290	200	90	3	1251†
	4	9.1	2.94	196	107	89	2	484
39	1	11.1	6.04	420	283	137	2	4287
	2		4.57	284	167	117	0	1709
	3		4.01	326	213	113	2	1831‡
	4	10.0	3.42	219	111	108	2	1190
45	1	21.5	5.20	309	94	215	3	901
	2		5.31	340	108	232	0	882
	3		4.87	481	229	252	0	1018§
	4	20.2	4.72	289	86	203	0	667

* 6.7 milliequivalents of cystine disulfoxide (907 mg.) were given orally.

† 6.7 milliequivalents of cystine disulfoxide (907 mg.) were injected (in ammonia) subcutaneously.

‡ 3.35 milliequivalents of cystine disulfoxide (454 mg.) were injected (in ammonia) intravenously.

§ 6.7 milliequivalents of cystine disulfoxide (907 mg.) were injected (in ammonia) intravenously.

The taurocholic acid figures, exclusive of the experimental day, show a definite downward trend, so that any rise on the experimental day should probably be considered significant.

With the exception of Dog 45, all the extra sulfur which was excreted in the urine after the animals were given cystine disulf-

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oxide had been oxidized to sulfate, and most of the extra sulfur from Dog 45 was in the form of sulfate. Dog 45 showed an unusual sulfur distribution throughout the experiment, for it excreted only about a third of the urinary sulfur in the oxidized fraction. No excretion of extra disulfide sulfur was observed with any of the dogs.

Table II gives the data from five subcutaneous injections of cysteine sulfinic acid. Necrosis was not observed after these

TABLE II

Urinary Sulfur Partitions and Taurocholic Acid Production Following Administration of Cysteine Sulfinic Acid to Fasting Bile Fistula Dogs

Each animal was fed 2.8 gm. = 6.7 milliequivalents of cholic acid daily.

Dog No.	Day	Weight	Total N	Total S	Sulfate S	Organic S	--SS-- S	Taurocholic acid
		<i>kg.</i>	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
33	1	8.7	4.86	239	207	32	6	1541
	2		3.86	212	183	29	4	951
	3		5.22	321	279	42	6	1967*
	4		4.61	230	197	33	3	588
	5	7.3	4.85	348	312	36	2	1285*
37	1	8.8	4.22	181	147	34	7	1083
	2		3.61	163	123	40	7	841
	3		4.59	322	270	52	8	1829*
	4		4.17	200	162	38	5	878
	5	7.3	3.97	292	228	64	4	1217*
43	1	16.4	8.90	379	242	137	2	4537
	2		4.96	291	194	97	3	1982
	3		4.93	379	275	104	6	2139*
	4	14.1	4.76	326	206	120	10	1540

* 6.7 milliequivalents of cysteine sulfinic acid (1.02 gm.) were injected (in water) subcutaneously.

injections. In each case the taurocholic acid output was increased on the day of the injection, indicating that the sulfinic acid may have been converted to taurine. Most of the extra urinary sulfur from the cysteine sulfinic acid appeared in the sulfate fraction. With this compound, however, small but definite amounts of extra organic sulfur were excreted after each injection.

Table III presents the values obtained for taurocholic acid excretion and urinary sulfur distribution after three feedings of

cysteic acid.² Striking increases were found for the taurocholic acid values. In fact, nearly all the sulfur recovered after feeding cysteic acid to Dog 29 was that found in the extra taurocholic acid. The extra taurocholic acid value for this experimental day

TABLE III

Urinary Sulfur Partitions and Taurocholic Acid Production Following Administration of Cysteic Acid to Fasting Bile Fistula Dogs

Each animal was fed 2.8 gm. = 6.7 milliequivalents of cholic acid daily.

Dog No.	Day	Weight	Total N	Total S	Sulfate S	Organic S	-SS-S	Taurocholic acid
		kg.	gm.	mg.	mg.	mg.	mg.	mg.
29*	1	10.5	Urine lost				Not run	1282
	2		4.12	192	149	43		625
	3		4.22	210	172	38		580
	4		3.38	146	115	31		206
	5		3.75	279	243	36		1175†
	6		4.17	214	180	34		1290
	7		3.99	190	158	32		819
	8		4.02	192	149	43		2714‡
	9		3.01	165	132	33		1556
	10	9.0	3.28	178	143	35		1390
31	1	13.4	5.64	458	409	49	3	2130
	2		4.69	323	269	54	4	1593
	3		6.34	417	333	84	10	1494§
	4		4.11	257	216	41	2	1493
	5		5.72	378	282	96	2	2697‡
	6	9.0	5.75	326	280	46	5	2079
33	1	8.8	3.91	163	126	37	Not run	1058
	2		3.35	170	135	35		817
	3		2.64	222	136	86		1754‡
	4	7.7	Urine lost					893

* 25 gm. of sucrose were fed daily to Dog 29.

† 6.7 milliequivalents of cystine (0.8 gm.) were given orally.

‡ 6.7 milliequivalents of cysteic acid (1.13 gm.) were given orally.

§ 6.7 milliequivalents of cystamine dihydrochloride (0.75 gm.) were given orally.

indicates a conversion of more than half the cysteic acid to taurine. With regard to the urinary sulfur values the dogs differed somewhat. In contrast to the findings with cystine disulfoxide and

* The cystamine values are taken from work previously reported (3).

cysteine sulfinic acid, the extra sulfur from cysteic acid was found chiefly in the organic, rather than in the oxidized fraction of the urinary sulfur. The highest proportion of oxidized extra sulfur was excreted by Dog 31, which showed 55 mg. of organic sulfur and 66 mg. of sulfate sulfur. Dog 33 excreted 51 mg. of extra organic sulfur and no sulfate sulfur, while Dog 29 excreted no extra urinary sulfur at all after the ingestion of cysteic acid. Administration of cystine to Dog 29 was followed by results such as have been reported before (2); namely, increased taurocholic acid and sulfate sulfur excretion.

DISCUSSION

Several workers (4) have observed that cysteic acid is not oxidized by the animal body. White, Lewis, and White (5) administered this compound to rabbits orally and subcutaneously and found that the relatively small amount of sulfur of cysteic acid which had been oxidized was presumably a result of activity of intestinal microflora. Such intestinal action may have been responsible for the urinary sulfate obtained from our dogs.

Medes (1) observed that the following series holds true both for rate of oxidation to inorganic sulfate and for urinary excretion of total sulfur: cystine disulfoxide > cysteine sulfinic acid > cysteic acid. She suggested that the sulfur which was not recovered in the urine might have been used for formation of taurine. The results we have obtained indicate that such utilization of these sulfur-containing substances is possible, although the total recovery of sulfur in urine and bile accounts for only two-thirds to three-fourths of that administered. A greater proportion of sulfur was recovered in our earlier experiments with cystine (2), reported in the first paper of this series. Since administration of cysteine sulfinic acid was followed by less conversion to sulfate than was administration of cystine disulfoxide, and since cysteic acid produced less sulfate than did the sulfinic acid, we cannot conclude that these substances represent the normal path of oxidation of cystine to sulfate.

Cystine disulfoxide, cysteine sulfinic acid, and cysteic acid all promoted the excretion of extra taurocholic acid. It is conceivable that this series of substances could be intermediates in the formation of taurine from cystine by the dog, since the substances

originally more highly oxidized appeared to give rise to more taurocholic acid. However, no claims to that effect can be made before such compounds are found to exist in the body.

It is of interest to note that under our conditions cysteic acid gave rise to taurine quite readily, while under the same circumstances cystamine (3) did not. Does this mean that decarboxylation of cystine occurs more easily *in vivo* than oxidation of its sulfur? The reverse relation seems to hold true *in vitro*, for taurine is easily produced from cystamine (6), while its preparation from cysteic acid is difficult (7).

SUMMARY

1. Cholic acid was fed to fasting bile fistula dogs for several days to deplete their livers of taurine by increasing their output of taurocholic acid. Cystine disulfoxide given orally or parenterally, cysteine sulfinic acid injected subcutaneously, or cysteic acid given by mouth with the cholic acid, usually on the 3rd day of the fast, increased the excretion of taurocholic acid. Cysteic acid was especially effective in this respect. It therefore appears that each of these three substances may be changed to taurine by the dog.

2. Nearly all the extra urinary sulfur from orally or parenterally administered cystine disulfoxide was found in the sulfate fraction of the urine. The greater part of the extra sulfur from subcutaneously injected cysteine sulfinic acid appeared as urinary sulfate. Very little of the sulfur of orally administered cysteic acid, however, was oxidized to sulfate.

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CATALASE. II

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In our paper (1) announcing the crystallization of beef liver catalase we stated that in addition to hematin this enzyme contained a second side chain. The existence of this was evident through the production of a strong blue color upon addition of hydrochloric acid to catalase solutions. In the present paper we include new observations concerning the preparation and properties of catalase. Most of our attention, however, is directed to an investigation of the blue substance and to the partition of iron between the hematin and the blue substance.

Crystallization of Catalase—We have observed that if the catalase crystals from the first crystallization are very fine, as often is the case, they will not pack well in the centrifuge tubes, and hence will contain considerable ammonium sulfate which was not removed from the mother liquor. The ammonium sulfate renders the crystals difficult to dissolve by the addition of alkaline phosphate buffer of pH 7.4. This difficulty can be overcome by stirring the crystals with a minimal amount of distilled water together with 5 cc. of 9.6 per cent alkaline phosphate buffer of pH 7.4 for every 5 pounds of liver used, and then dialyzing against distilled water until the crystals have gone completely into solution and nothing but denatured protein remains. The bulk of this denatured protein may be rather large if the tubes become too warm during the centrifuging. The dialysis described above has never been found necessary for catalase after the first crystallization.

Another point of interest is that crystalline catalase may be prepared in the following manner without the use of ammonium sulfate. Extract 300 gm. portions of finely ground beef liver with

400 cc. portions of 35 per cent dioxane, and filter overnight in the ice chest. Add 20 cc. of dioxane to every 100 cc. of filtrate, let stand 12 to 24 hours in the ice chest, and filter again. Next add 10.2 cc. of dioxane to every 100 cc. of filtrate and allow to stand in the ice chest for 24 hours. Filter, allowing to drain completely. Now scrape the precipitate from the filter paper and stir with water to form a thin cream. Add a few drops of saliva to digest the glycogen. Filter, place the filtrate in a collodion sac, and dialyze against distilled water. The catalase precipitates as beautiful prisms and sometimes partly as plates. Photographs of these have been shown in our first paper (1). The catalase crystals thus obtained are undoubtedly of high purity, although the yield is somewhat diminished. To dissolve the crystals, one adds a very small amount of alkaline phosphate buffer of pH 7.4 and then solid sodium chloride is cautiously added until solution is complete. Since the crystals dissolve slowly, a sufficient time must elapse after every addition of the sodium chloride. After filtering, the material can be recrystallized by dialyzing and adding a drop of saturated potassium dihydrogen phosphate from time to time if necessary. An alternative procedure is simply to neutralize the alkaline sodium chloride solution by adding $2\frac{1}{2}$ volumes of saturated potassium dihydrogen phosphate for every volume of alkaline phosphate used, and then chilling the material in the ice box. By this means catalase precipitated in the form of plates or prisms is always converted to the needle form, described in our first paper. The needle form is soluble in alkaline phosphate buffer of pH 7.4, whereas the prism or plate forms require the addition of sodium chloride. In this conversion of prisms or plates to needles, the needles will begin to crystallize from the alkaline phosphate-sodium chloride solution without neutralizing or change of temperature, if too much sodium chloride or alkaline phosphate has been used. It is of interest also to note that a very strong solution of catalase made by dissolving the needle form in alkaline phosphate buffer of pH 7.4 will soon begin to deposit prisms or plates or both without change in temperature or neutralization.

Activity of Catalase—Von Euler and Josephson (2) found a *Kat. f.* of 43,000 for one sample of their purified horse liver catalase. We reported in our first paper (1) an average value of 26,000 for beef liver catalase which had been recrystallized several times.

Since this time, we have occasionally found values as high as 35,000. For example, we give as illustration the figures in Table I for a sample of catalase crystallized first as prisms according to the method outlined above, and then recrystallized three times as needles. We believe that variations in the activity reported for crystalline beef liver catalase are to be ascribed to some failure to control completely the experimental conditions in the determinations. The results obtained by ultracentrifugation and diffusion in Svedberg's laboratory (3) have shown our enzyme to be essentially pure.

Agner (4) obtained a horse liver catalase of *Kat. f.* 55,000 to 60,000, but states that the ultracentrifuge showed this to contain from 15 to 20 per cent of impurity. Agner claims, furthermore, that he demonstrated to one of us (Professor Sumner) that analyses run on crystalline beef liver catalase by him gave a result strictly comparable to our results. He then gives the *Kat. f.* of our product as 28,000. This is an unfortunate statement, since the figure obtained by Agner¹ for beef liver catalase was actually 38,000 instead of 28,000.

Stern and Wyckoff (5) give as the *Kat. f.* of their best preparation a value of 33,448. Since their enzyme was prepared by sedimentation in the ultracentrifuge, it was presumably in reasonably pure condition. These authors, however, make the rather surprising statement of having failed in the preparation of crystalline beef liver catalase. Many of our graduate students have prepared crystalline catalase without difficulty. There have been no failures so far.

Does Beef Liver Catalase Contain Copper?—Agner (4) states that horse liver catalase contains copper, and that this metal may be

¹ The figures taken down from Agner as he gave them were:

Time	KMnO ₄	Dilution of catalase solution	Solids in catalase solution by dry weight determination
min.	cc.		per cent
0	3.98	0.02:200 cc. (0.5 cc. of this used in analysis)	1.12, 1.14
3	3.43		
5	3.10		
10	2.59		

present as a part of the catalase molecule. Dr. Eusebio Tria, working in this laboratory, finds by analysis of the ash from thoroughly dialyzed catalase recrystallized three times that the percentage of copper is not more than 0.0015, and hence that not more than 0.056 atom of copper is present per molecule of catalase. The method used for copper analysis was that of Tompsett (6). The dry weight of catalase used was 0.1022 gm.

Absorption Bands of Catalase—Zeile and Hellström (7) reported absorption bands for horse liver catalase at 629, 540, and 500 $m\mu$. Stern (8) reported bands in the visible spectrum at 622,

TABLE I
Determination of Activity of Catalase

Dilution of catalase		k	Weight of 1 cc. undiluted catalase dried at 100°	Weight of ash	Kat. f.
	min.		gm.	gm.	
1:10,000	0	0.0510	0.0429	0.0006	35,700
	3	0.0493			
	6	0.0471			
	9	0.0453			
1:20,000	0	0.0247	0.0429	0.0006	34,500
	3	0.0243			
	6	0.0237			
	9	0.0220			

The k values used for the calculation of Kat. f. were obtained by plotting the k values for 3, 6, and 9 minutes and extrapolating to the value at zero time. The k values at 3 minutes also can be used without much difference in the Kat. f. values.

540, and 505 $m\mu$, for partially purified horse liver catalase. Keilin and Hartree (9) reported bands at 629.5, 544, and 506.5 $m\mu$ for a catalase preparation from horse liver. We have already reported that crystalline catalase shows bands at 627 and 536 $m\mu$. We have found the third band at approximately 502 $m\mu$; this is rather broad and difficult to see by illumination from our tungsten filament lamp. It is rather plainly visible, however, with the pocket spectroscope and direct sunlight as a source of illumination.

Iron Content—We previously reported the iron content of beef catalase as about 0.1 per cent, although some of our values were as

low as 0.06 per cent. We now believe that the low results were erroneous, owing to failure to dissolve all of the iron in the ash. Our improved method shows rather consistent values of from 0.09 to 0.10 per cent.

Second Side Chain of Catalase—As we have already reported, upon addition of hydrochloric acid to crystalline catalase, the coagulated protein turns a strong blue color. Glacial acetic acid dissolves the protein and yields a blue solution. A small sample of lamb liver catalase, obtained with great difficulty, also gave this blue color. Addition of excess of acetone causes the blue substance as well as the hemin to dissolve from the precipitated protein. The blue solution at this point shows absorption bands at 643, 543, and 507 μ , which, of course, are due to the hemin. By using methyl alcohol or ethyl alcohol blue solutions also can be obtained. Dioxane gives a blue-green solution.

The hemin can be removed from the blue solution in a quantitative manner by evaporating in a vacuum until nearly all of the acetone is removed. The crystals of hemin that have formed are then centrifuged down and the remaining blue solution is decanted.

The blue color of the aqueous solution gradually fades upon standing. The effect of alkali is to turn it brown; reacidification results only in partial regeneration of the blue. In the ice box, a precipitate gradually forms; this can be filtered off and dissolved in acetone plus a few drops of *N* hydrochloric acid. The ash from this green solution shows a low content of iron, whereas most of the iron remains in the filtrate. A part of this iron is in the ionic form, but more work will be necessary to determine whether it is all ionic.

Shaking with chloroform removes the blue substance from water, giving a green chloroform solution. If the chloroform solution is shaken with aqueous alkali, the substance is removed from the chloroform.

The blue material resembles bilirubin in giving a Gmelin test with Hammarsten's reagent.² Here the blue is changed to violet

² We prefer a more concentrated reagent than the original. We add 16 cc. of concentrated nitric acid to 50 cc. of concentrated hydrochloric acid. When this mixture has become yellow, we add it to 300 cc. of 95 per cent ethyl alcohol.

and then to red. These substances show absorption bands similar to the bands formed by treating bilirubin with Hammarsten's reagent.

It has been observed previously by Stern (10) that crude catalase preparations from horse liver yield this blue-colored solution containing hemin, upon addition of acetone and hydrochloric acid. Stern claimed the substance to be biliverdin, occurring as an impurity. Lemberg (11), on the other hand, mentions that "green hemin" or "verdohemochromogen" accompanies the catalase of horse liver extracts. Later Stern and Wyckoff (5) speak of the blue substance as verdohemochromogen.

As we have stated previously, this blue substance from crystalline beef liver catalase is not biliverdin, and we have no convincing evidence that it is verdohemochromogen, although it is somewhat similar to both substances. It cannot be removed from the catalase by repeated recrystallizations; indeed, it rather increases with the purity of the enzyme. The blue substance is not present as such in the catalase molecule, but is produced upon the addition of strong acid to the catalase.

Isolation of the Blue Substance—Catalase was prepared from 18 pounds of beef liver and was recrystallized three times, yielding about 1.5 gm. This was found to have a *Kat. f.* of 30,000 after standing 1 month in the ice chest. The third recrystallization was brought about by dialysis, which gave a product practically free from inorganic salts. This material was centrifuged down and was suspended in a volume of 25 cc. of redistilled water.

For isolation of the blue substance, duplicate preparations were made; 5 cc. of the well suspended crystals were used in each. The 5 cc. of crystalline material were run slowly into 50 cc. of acetone plus 10 cc. of *N* hydrochloric acid, with constant stirring. The material was then filtered and the protein residue was washed with acetone until free from extractable color. The acetone solutions were combined and were evaporated at about 30° in a pear-shaped Claisen flask in a vacuum from a water pump. When the volume of the residue indicated that the amount of acetone used had nearly all evaporated and largely water and solids remained, the evaporation was discontinued and the crystals of hemin were centrifuged down. The blue substance remained in the aqueous layer. From spectroscopic examination, and from previous trials

of the pyridine hemochromogen test on similar samples, we judge the removal of hemin to be quantitative. Solution of the centrifuged hemin in acetone gave a perfectly clear brown solution with no visible blue or green color.

In the second preparation, the evaporation of acetone was carried a little too far, so that some of the blue material precipitated with the hemin. For this reason, it was necessary to dissolve the hemin in acetone, add about 3 cc. of water, and evaporate again to precipitate the hemin without the blue substance. The hemin was again centrifuged down and the blue solution was added to the first blue aqueous solution. Apparently a small amount of acetone must be present to prevent precipitation of the blue material.

As shown in Table II, the amount of iron in the hemin is approximately equal to the amount in the blue substance. Hence

TABLE II
Partition of Iron in Catalase

Experiment No.	Catalase	Fe in protein residue	Fe in hemin	Fe in blue substance	Hemin calculated from Fe	Hemin by colorimetry	Total Fe in catalase	Total Fe by adding fractions
	mg.	mg.	mg.	mg.	per cent	per cent	per cent	per cent
1	256	0.027	0.119	0.102	0.54		0.92	0.96
2	256	0.025	0.100	0.116	0.46	0.42	0.92	0.94

it is not possible to calculate the hemin content of catalase from the total iron. The per cent of hemin calculated from the hemin iron is from 0.46 to 0.54, while a direct determination of the isolated hemin dissolved in acetone and compared with a hemin standard gave 0.42 per cent.³ Hence the beef liver catalase molecule, with about 0.5 per cent hemin, 0.1 per cent iron, and a molecular weight of 248,000 (3), contains not 4 but only 2 molecules of hematin. Either the claim made by Stern and Wyckoff (5) that horse liver catalase contains 4 molecules of hematin is erroneous, or else horse liver catalase has twice as much hemin as beef catalase.

Attempts to Separate the Side Chains of Catalase Reversibly—We have attempted to split the side chains from catalase in a rever-

³ Zeile and Hellström (7) reported a hemin content of 0.6 per cent in horse liver catalase.

sible manner, as Theorell (12) has succeeded in doing with the yellow oxidation enzyme. However, upon dialyzing against 0.02 N hydrochloric acid, the catalase became partly denatured and partly inactivated, apparently without decomposing into protein and side chain fractions. When a solution was stirred with sufficient methyl or ethyl alcohol to produce a precipitate, no splitting of the catalase into protein and prosthetic groups could be brought about unless the alcohol contained acid. If traces of acid were present, the catalase was decomposed to a slight extent, giving a filtrate faintly brown in color. The brown filtrate contained traces of hematin and produced a little blue color upon addition of hydrochloric acid.

We observed that a solution of crystalline catalase is inactivated by Fairchild's trypsin after standing for a day or more at 37°. If the digest is dialyzed, a brown substance passes through the colloidion membrane. At this point the addition of acetone and hydrochloric acid produces a blue color neither with the material inside the membrane nor with the solution outside.

SUMMARY

1. Modifications of the method for preparing crystalline beef liver catalase are described.

2. Beef liver catalase is shown to be essentially free from copper.

3. The total iron of beef liver catalase is between 0.09 and 0.10 per cent.

4. Catalase contains two side chains. One of these appears as hemin, while the second is split off as a blue-colored substance upon the addition of acetone and hydrochloric acid.

5. Only one-half of the iron in catalase is present in the hemin, and consequently beef liver catalase contains only 2 hemin molecules per molecule of catalase.

6. Treatment of catalase with slightly acid alcohol removes a small part of the prosthetic material, giving a brown solution. This contains hematin and turns blue on acidification.

7. Digestion of crystalline catalase with trypsin inactivates the enzyme and prevents subsequent production of blue substance.

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CATALYTIC EFFECTS OF POROUS POWDERS ON PURE VITAMIN A

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In this laboratory we had often observed the development of orange- or red-colored bands while filtering solutions of fish liver oil concentrates through adsorption (Tswett) columns of porous powders. Other workers reported similar observations.

Castle, Gillam, Heilbron, and Thompson (1), in 1934, while filtering solutions of fish liver oil concentrates through columns of alumina and of calcium hydroxide, observed both orange- and red-colored bands which they supposed might be due to some chemical change in the vitamin A present.

Karrer, Walker, Schöpp, and Morf (2), in 1933, while trying to isolate vitamin A, saw similar banding in their adsorption columns. By the use of calcium oxide or calcium hydroxide tubes the main portion of their vitamin A concentrate was separated into two fractions designated as α and β , with absorption bands of 270 and 580 $m\mu$ for the α , 328 and 620 $m\mu$ for the β .

Gillam and El Ridi (3), in 1935, claimed that chromatographic adsorption may cause isomerization of carotenoids and that by it they have converted β -carotene into α -carotene and *vice versa*. Gillam, Heilbron, Jones, and Lederer (4), in 1938, suspected a greater catalytic effect of alumina than of calcium hydroxide on certain of the substances present in a fish liver oil concentrate.

It seemed to us that the adsorbents were not actually separating orange or red substances already found in the liver oils but actually creating them by catalysis. Banding followed catalysis. One of us (H. N. H.) with the assistance of Miss Eva Hartzler made some tentative tests of this idea 2 or 3 years ago, using approximately 50 per cent vitamin A concentrates. Our suspicions were strengthened even though we were not working with

the pure vitamin. Obviously the question as to the origin of the orange or red substances could be settled only by treatment of solutions of pure vitamin A with various adsorbents. After the isolation (5) of crystalline vitamin A in this laboratory, experiments became possible. Combinations of seven solvents for the vitamin and seven solids in powder form were tried. The chemical changes produced in pure vitamin A were sometimes astonishing and served as a warning against blind use of adsorption columns with sensitive substances.

EXPERIMENTAL

Activation of Adsorbents—All adsorbents used were activated for 2 hours at 200° in a stream of dry nitrogen, immediately before use. A rough preliminary experiment, in which vitamin A solutions in all seven solvents were treated with each of the adsorbents, was used to indicate which adsorbent-solvent combinations would prove most interesting.

Preparation of Solutions—Volumes of methanol solution (0.75 cc. containing 30 mg. of vitamin A) were measured into nitrogen-filled test-tubes (12 mm. in diameter) and the methanol removed almost completely by evaporation at room temperature in a vacuum desiccator. Exactly 0.5 cc. of the desired solvent was then added to each tube and, after the vitamin was dissolved, enough of the activated adsorbent was added to form a "mud" with the solution. In other words, there was little or no clear supernatant liquid. Deep colors sometimes developed within a few minutes, but, when the colored material was soluble in the solvent (*e.g.* "activated alumina" with acetone solution), the increase in depth of color required several hours. The tubes were allowed to stand for about 20 hours at room temperature at which time the vitamin A and contaminating products of catalysis were eluted by washing three or four times with 0.75 cc. of appropriate solvent, centrifuging, and decanting after each elution. The results of the tests on these filtrates are given in Table I. The blanks were treated in the same way as the other tubes except that after addition of the 0.5 cc. of solvent no adsorbent was added, the tubes merely being stoppered and allowed to stand 20 hours. The direct color of the filtrate was read in a Lovibond tintometer through the thickness of the test-tubes. The results

given in Table I were obtained by correcting the figures actually read to a basis of 10 mg. of solid per cc. of solution. For example, if 1 cc. contained 20 mg., the reading in color units was divided by 2.

Determination of Blue Value—The blue values were determined with the standard antimony trichloride test. They cannot be regarded as very accurate because the relative humidity of the room at the time was too great (about 60 per cent usually), but they can be compared relatively.

Determination of $E_{1\%}^{1\text{cm}}$ Value—The spectroscopic absorption was measured in a Hilger vitameter, with silver electrodes for the arc. The accuracy of the instrument is not equal to that of the complete spectrophotometer. Furthermore, values of vitamin A change on standing in various solvents at room temperatures.

Solvents—Acetone, Baker's Analyzed, c.p.

Methanol, from Commercial Solvents Corporation, their 99 per cent grade, distilled to remove non-volatile residue.

Ether, Merck's Reagent, absolute.

Pentane, b.p. 40–45°.

Chloroform, from the Dow Chemical Company, u.s.p. grade.

Benzene, Baker's Analyzed, c.p., thiophene-free.

Cyclohexane, from Eastman Kodak Company, Practical, purified by fractional crystallization.

Adsorbents—Alumina, "activated alumina" from the Aluminum Company of America.

Alumina, Merck's aluminum oxidatum.

Silica gel, Patrick's, from the Silica Gel Corporation, not freed from iron.

Silica gel, Patrick's, freed from iron by acid treatment.

Silica gel, Holmes' chalky type (6). Free from iron.

Magnesium oxide, micron brand from the California Chemical Company.

Calcium hydroxide, Baker's, u.s.p.

Fullers' earth, uncertain source.

Lloyd's Reagent, from Eli Lilly and Company.

Adsorbate—Vitamin A, our purest product, recrystallized several times from methanol.

Ionone, Eimer and Amend's 100 per cent, probably a mixture of isomers.

Results

Distortion of Vitamin A Readings—In the other solvents not included in Table I it is noteworthy that magnesium oxide raised the $E_{1\text{cm}}^{1\%}$ readings and the antimony trichloride "blue value" from 10 per cent to 20 per cent (except in chloroform, where both readings fell approximately 40 per cent below that of the control solution).

Calcium hydroxide showed a similar loss of 40 to 50 per cent in chloroform but caused only moderate apparent increase in methanol and acetone. In cyclohexane the $E_{1\text{cm}}^{1\%}$ loss was 38 per cent.

Merck's alumina was satisfactory with methanol but not in some other solvents. "Activated alumina" was satisfactory in methanol, ether, and chloroform, but not in acetone or cyclohexane.

Lloyd's Reagent and a commercial silica gel containing a little iron oxide were ruinous, as shown by the great lowering in vitamin A readings whenever these powders were used.

Color Changes—Judging from the increase in yellow color of the filtrate and residues (and appearance of red), cyclohexane is the poorest of the solvents tried. Ether and benzene both favor color development.

Among the solids, "activated alumina" always developed more color than did Merck's alumina. Calcium hydroxide caused little or no color change in benzene or chloroform but caused a very large color increase in methanol, ether, cyclohexane, and pentane. Magnesium hydroxide caused a large increase in color with ether, acetone, and cyclohexane, a moderate increase with methanol and benzene, but no increase in chloroform. Purified silica gels were not color developers.

"Activated Alumina" Column Experiment—It seemed desirable to apply the findings of the above catalytic experiments to an actual adsorption column. A solution of 0.25 gm. of vitamin A in 5 cc. of a pentane-benzene mixture (4:1) was passed through a column of "activated alumina" (100 to 150 mesh) 12 cm. deep and 1.7 cm. wide. Three colored bands appeared and were developed by washing with 300 cc. of the pentane-benzene mixture. The top band, about 1 cm. wide, was yellow, becoming more orange at the lower edge. The next was a 2 mm. wide, dark brown band, and the lowest a

TABLE I
Effect of Various Adsorbents on Vitamin A in Pentane or Benzene

Adsorbents	Color of filtrate	Color of residue on evaporation of filtrate	Desorbing solvent	SbCl ₅ blue value of filtrate	E 1% value of filtrate
In pentane					
None	9 yellow	Yellow		45,000	1040
Activated alumina	40 "	Red	MeOH	82,000	1590
Merck's Al ₂ O ₃	13 red				
Silica gel, Holmes'	15 yellow	Yellow	Pentane	67,000	1420
SiO ₂	14 "	"	MeOH	70,000	1240
Silica gel (pure), Patrick's	16 "	"	"	55,000	1180
Silica gel (Fe ₂ O ₃ impurity), Patrick's	55 " 2 red	" oil and white powder	"	8,600	650
MgO	45 yellow, 3 red	Orange	Pentane	73,000	1510
Ca(OH) ₂	35 yellow, 3.5 red	Orange-red	"	79,000	1480
In benzene					
None	19 yellow	Yellow		62,000	1230
Activated alumina	43 " 25 red	Red	MeOH	80,000	1720
Merck's Al ₂ O ₃	21 yellow	Yellow	"	49,000	1030
Silica gel, Holmes' SiO ₂	24 "	Yellow-orange	"	55,000	1450
Silica gel (pure), Patrick's	25 "	Yellow	"	61,000	1440
Silica gel (Fe ₂ O ₃ impurity), Patrick's	51 " 1 red	" oil and powder	"	3,200	450
MgO	39 yellow, 2.2 red	Orange	C ₆ H ₆	56,000	1300
Ca(OH) ₂	13 yellow	Yellow-orange	"	59,000	1240
Fullers' earth	100 "	Orange oil, yel-	MeOH	3,000	490

band about 3 mm. wide, red-brown in color. The column was cut and the bands separated as well as possible into sections for elution with methanol. The fractions are further characterized in Table II.

Carotene—In earlier work on adsorption of carotene we occasionally obtained a greater depth of color in the desorbed material than the original solution. In the light of the present work this apparently points to a catalytic formation of more coloring matter. Gillam and his associates are cited on this point (4).

Ionone—Since ionone has the same ring structure as vitamin A, an experiment was planned to discover whether the adsorbents and solvents which were so catalytically active on vitamin A would produce any visible changes in ionone. Solutions of ionone (25 mg. per 0.5 cc.) in the seven solvents used for vitamin A experiments were treated with the activated adsorbents. None of the adsorbents produced colored materials from methanol or acetone solutions of ionone, but "activated alumina" produced an orange color with ionone in the other solvents, and also in the absence of solvent. The colored product was desorbed with acetone and the color of the solution measured in a Lovibond tintometer. Benzene favored production of the most highly colored product, 1 cm. depth of a solution containing 6 mg. per cc. reading 2.0 yellow units in the tintometer. Ionone itself is colorless. Less intense colored materials were produced in ether, pentane, and chloroform solutions with Patrick's crude silica gel, in benzene and cyclohexane solutions with magnesia, and in cyclohexane solutions with calcium hydroxide. Faintly yellow materials were obtained from pentane solutions with magnesia and calcium hydroxide, and from ionone in the absence of solvent with Patrick's crude silica and with calcium hydroxide.

DISCUSSION

The experimental results certainly indicate the desirability of preliminary tests before filtering vitamin A solutions through adsorbent columns. Since some combinations of solvent and adsorbent cause destructive changes in the vitamin, caution is desirable in assumptions that any particular substance is separated by column analysis into α , β , etc., forms. This separation may occur and yet it may be that a catalyzed product tenaciously adsorbs more or less of the original substance.

For that matter, the same tests and precautions must be advisable for other sensitive substances than vitamin A, if adsorption columns are used.

It is noteworthy that aluminas prepared by two distinctly different methods often act quite differently on vitamin A. Of the three silicas used, the two free from iron reacted in similar manner, but Patrick's commercial product containing a little ferric oxide impurity was usually devastating in its attack on the vitamin.

The extinction coefficient ($E_{1\text{cm.}}^{1\%}$) was often much higher after catalysis than in the blank 20 hours old. Of course this serves to remind us that other substances than vitamin A strongly absorb light of 328 $m\mu$ wave-length. Such substances occur in certain fish liver oils. Obviously, if such substances can form during

TABLE II
Fractions Obtained from "Activated Alumina" Column

Fraction	Blue value	$E_{1\text{cm.}}^{1\%}$ value	Blue value E value	Color of residue
Top, yellow	58,000	1150	50	Orange and yellow oils
Next, orange	71,000	1310	54	Red and yellow oils
Mixture of brown and dark red	68,000	1520	45	Dark red oil
Darkest red	59,000	1270	46	Red waxy "

column treatment, the extinction coefficients of some concentrates will read too high. Similar alarming variations are observed from the blue values in Tables I and II.

It is now evident from the experiments with magnesium oxide that some earlier high blue values of vitamin A concentrates reported from this laboratory were, in part, due to catalytic action of magnesia columns on the vitamin or, possibly, on associated impurities.

SUMMARY

Solutions of pure vitamin A in seven different solvents were treated with seven different adsorbents in order to aid in selection of safe adsorbent columns. Catalytic formation of new substances, sometimes orange or red, sometimes with greatly changed blue values and extinction coefficient values (328 $m\mu$), was observed in several instances.

It is shown that preliminary testing for such catalysis is advisable before subjecting any very sensitive substance to treatment with adsorption columns.

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THE INFLUENCE OF SOME INTERMEDIARY METABOLITES AND SALTS ON THE RESPIRATION OF LIVER TISSUE SUSPENSIONS

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The respiration of slices of kidney cortex is considerably increased by the addition of a series of compounds—pyruvate, succinate, fumarate, malate, oxaloacetate—and these substances are used up at a rate which would account for more than the increased oxygen consumption as well as for certain amounts found to be synthesized into carbohydrate (1-3). It was suggested that the main course of the oxidative metabolism of these substances in kidney cortex is a cycle of reactions whereby one substance is converted successively into the next in the order given above. This theory may need amplification in view of recent work on citrate and α -ketoglutarate with minced tissues (4), and in view of the fact that these two compounds behave similarly to the above compounds with kidney slices.¹ In all sliced tissues other than kidney so far tried, namely rat liver, brain, testis, and cancer tissue (2, 5), and ox retina and chick embryo (6), there was no evidence that a similar cycle of reactions takes place, since fumarate and malate are scarcely, if at all, oxidized.

Lately it has been shown that most tissues contain malic dehydrogenase (7) and that fumarate and malate promote catalytically the respiration of minced tissues (4, 8-10).

The relation between the processes involved in the oxidative removal of these substances in kidney slices, their lack of effect with other sliced tissues, and their catalytic behavior as found in minced tissues are obscure. The following observations on the effects of various metabolites on the respiration of rat liver sus-

¹ Elliott, K. A. C., and Greig, M. E., unpublished data.

pensions were made while information was being collected for the solution of this problem. Comparative studies on other tissues will be reported later.

EXPERIMENTAL

General Methods—The simple Barcroft differential respirometer (11) was used for most of these experiments. The center tube contained 0.3 cc. of 8 per cent KOH and a splayed roll of Whatman No. 40 paper to absorb CO_2 (12). The vessels were in a bath at 37° . Since large O_2 uptakes occurred, it was necessary occasionally to open the manometer stop-cocks to the air to allow the manometer fluid to return to level. The stop-cocks were closed again after 30 seconds and readings continued. The manometers were shaken at 132 to 150 oscillations per minute with a 2.5 cm. excursion. Raising the rate of shaking did not increase the rate of oxygen uptake.

Measurements of R.Q. were made by the method outlined by Dixon and Elliott (13). With one manometer, A, the O_2 uptake was measured as usual. Two other manometers, B and C, contained the same additions but no alkali papers. Hanging tubes, containing 0.3 cc. of 3 N HCl, were tapped into Manometer Vessels B and C at the end and at the start of the experimental period respectively. If h_A is the reading of Manometer A at the end of the experimental period, and h_B and h_C the readings of Manometers B and C when they have become constant,² and $k_{\text{O}_2}^A$, $k_{\text{CO}_2}^A$, etc., are the vessel constants, then

$$\text{O}_2 \text{ uptake} = h_A k_{\text{O}_2}^A$$

$$\text{CO}_2 \text{ evolution} = h_B k_{\text{CO}_2}^B - h_A \frac{k_{\text{O}_2}^A \cdot k_{\text{CO}_2}^B}{k_{\text{O}_2}^B} - h_C k_{\text{CO}_2}^C$$

² When the acid is added to Vessels B and C, the CO_2 absorbed by the medium is liberated rapidly. There often follows a very slow continuous further evolution of gas, probably CO_2 resulting from the decomposition of compounds such as acetoacetic acid. The figures for h_B and h_C were obtained by extrapolation back to the time the acid was added. If the readings taken 75 minutes after the addition of the acid were used for the second experiment of Table IV, the R.Q. values would have been 0.76, 0.85, 0.90, instead of 0.78, 0.88, 0.93.

For experiments in bicarbonate-buffered medium the Dixon-Keilin apparatus (11) filled with oxygen containing 5 per cent CO_2 was used. The tissue suspensions were made by homogenization, with the apparatus of Potter and Elvehjem (14). Unless otherwise stated, the liver was removed from a decapitated rat and cooled over ice; excess blood was drained off with filter paper and 4 gm. weighed into the homogenizer tube. This was thoroughly disintegrated in, and made up to 14 cc. with, 0.05 M sodium-potassium phosphate buffer, pH 7.4. The tube was cooled in ice water during the homogenization. The suspension was used at once. The manometer vessel received 1 cc. of the suspension, containing 286 mg. of moist tissue, and the total volume of fluid in the vessel including other additions and water was 3 cc.

The solutions used were as follows: NaCl, NaBr, NaNO_3 , Na acetate (pH 7.4), 0.42 M; Na_2SO_4 , 0.345 M; $\text{Na}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$ (pH 7.4), 0.353 M; glucose, 0.775 M. These solutions are all approximately isosmotic, and 1 cc. of any, diluted with 1 cc. of 0.05 M phosphate buffer and 1 cc. of water, gives a solution isotonic with serum. *l*-Malate, citrate, α -ketoglutarate, pyruvate, tartrate, and acetate were prepared by neutralizing the acids to pH 7.4 with NaOH and making up to 0.21 M; *dl*-lactate 0.42 M. Oxaloacetic acid was dissolved, neutralized in dilute solution, and made up to 0.021 M immediately before use.

Results

Malate and Salt Effects—The effect of fumarate in increasing respiration of minced pigeon breast muscle has been studied by various authors (4, 9, 10, 15–19). Stare (20) showed that fumarate preserved the initial respiration of minced liver. Additions of either fumarate or malate give the same effects, since most tissues show very active fumarase activity (21), establishing rapidly an equilibrium mixture of the two substances. Tables I and III and Fig. 1 show that with homogenized liver, in the presence of NaCl, added malate causes not only the maintenance of the respiration rate as observed by Stare but also an acceleration of the initial respiration. The initial respiration with added NaCl, but no malate, is often constant for nearly 20 minutes after the equilibration period, but it is lower than when malate is added and always falls off some time earlier.

It has been realized by most workers who have studied fumarate effects that the composition of the medium has a considerable effect on the results observed. Straub and Annau (22) found the respiration of minced muscle, liver, and kidney was lower in Ringer-phosphate medium than in plain 0.067 M phosphate buffer and the fumarate effect was much less marked. These results of Straub and Annau were almost certainly due to the presence of Ca in their Ringer-phosphate. Greville (18) showed that the addition to the medium of 0.002 M CaCl_2 (about the concentration in blood or Ringer's solution) caused minced pigeon muscle to show a low respiration which was little stimulated by fumarate. Stare and Baumann (10) found that stronger effects were obtained with pigeon breast when a Ringer-phosphate medium (containing no Ca) was used instead of 0.067 M phosphate buffer, and Greville (18) considered that the effects were dependent on the osmotic pressure of the medium. Greville found that 0.05 M NaCl raised and maintained the respiration of pigeon breast mince. Fumarate, in 0.02 M concentration which is approximately isotonic with 0.03 M NaCl, had a similar effect, so he considered that at least part of the fumarate effect on muscle respiration was due to its osmotic pressure. Krebs and Eggleston (9) also observed the inhibitory effect of Ca on minced pigeon muscle and kidney. They did not observe the necessity for NaCl probably because their buffer was prepared from Na_2HPO_4 and HCl and so contained sufficient chloride for muscle respiration.

The results here reported show extremely marked effects of NaCl on liver suspensions, which are not all simply osmotic pressure effects.

In Tables I and III the rates of respiration during the first and second 10 minute intervals are given to show constancy or falling off in the initial period. For each series of experiments the total O_2 uptake is usually given at the time when the respiration of the most active member has begun to fall off.

The effects of salts on the respiration of liver suspensions with and without added malate are shown in Table I.

Adding 0.1 cc. of malate solution to the liver suspension (prepared as described above) caused no change in the low rate of respiration. When 0.1 cc. of NaCl solution ($\text{NaCl} = 0.014 \text{ M}$) was added, there was still no change in respiration with or without added malate.

When 0.3 cc. of NaCl was added, there was a large increase in respiration rate and the addition of 0.1 cc. of malate caused a further increase in initial rate. With 0.6 cc. of NaCl (0.084 M) the respiration was still further increased and added malate caused further initial increase which continued for a longer period than with 0.3 cc. of NaCl. With 1 cc. of NaCl, giving a medium isotonic with serum, the effects with and without malate were somewhat depressed again. The optimum NaCl concentration appeared to be about 0.08 M.

In order to test whether the effects were due to osmotic pressure, glucose solution was tried. With 0.3 cc. of glucose there was little effect on respiration; 0.6 cc. and 1.2 cc. increased respiration considerably for a very short time and to about the same extent. Apparently, therefore, osmotic pressure is a factor in determining the residual respiration; but in low concentration glucose is less effective than NaCl and the respiration falls off again even more rapidly than with NaCl. The osmotic pressure does not appear to determine the malate effect. With 0.3 cc. of glucose, added malate, 0.1 cc., caused a fleeting increase in respiration, probably due to its osmotic pressure. With 0.6 cc. and 1.2 cc. of glucose, added malate had almost no effect.

Neither sodium sulfate nor phosphate buffer increased the respiration or produced the malate effect appreciably. Actually they inhibited the effect of added chloride.³

However, NaCl could be replaced by NaBr and by NaNO₃ with almost identical results. Acetate behaved anomalously since it did not increase residual respiration, but, with added malate, the respiration was as high as with NaCl plus malate. It thus seems that univalent anion is specifically required for the production of the malate effect and also these anions (with the exception of acetate) improve the residual respiration more readily than does iso-

³ If the liver is homogenized in water instead of phosphate buffer, the malate and NaCl effects are shown in the absence of buffer. But if 1 cc. of 0.05 M phosphate buffer is now added to the aqueous suspension in the manometer vessel (making 0.017 M phosphate), the effects of both NaCl and malate are no longer shown. It was mentioned above that high concentrations of added phosphate inhibit the effect of small amounts of NaCl. But it is not understood why 0.017 M phosphate, added after homogenization, should inhibit, when tissue actually homogenized in 0.05 M phosphate is completely active though the final phosphate concentration in the experiment is nearly the same, 0.012 M.

TABLE I
Effects of Salts on Respiration of Liver Suspensions with and without Added Malate

286 mg. of moist tissue in 3 cc. of 0.012 M phosphate buffer, pH 7.4. 1 cc. of any solution in the 3 cc. makes the medium approximately isotonic with serum. 0.1 cc. of malate solution makes the concentration in the medium 0.007 M. The figures give the c.mm. of O_2 uptake.

	No NaCl		0.1 cc. NaCl		0.3 cc. NaCl		0.6 cc. NaCl		1.0 cc. NaCl	
		0.1 cc. malate		0.1 cc. malate		0.1 cc. malate		0.1 cc. malate		0.1 cc. malate
First 10 min.....	51	52		152	99	152	159	196	131	151
Next 10 ".....	43	44		73	65	73	146	191	130	156
Total in 110 min.....	236	235			280	395	635	1054	406	793
First 10 min.....	52	54					152	201	130	163
Next 10 ".....	47	40					83	192	69	157
Total in 100 min.....	192	194					352	797	316	634
	No addition		0.3 cc. glucose		0.6 cc. glucose		1.2 cc. glucose		0.6 cc. glucose	
				0.1 cc. malate		0.1 cc. malate		0.1 cc. malate		0.1 cc. malate + 0.4 cc. NaCl
First 10 min.....	53	57					129			
Next 10 ".....	41	45					73			
Total in 65 min.....	182	185					312			
First 10 min.....		83		143		137	142	149	174	167
Next 10 ".....		51		67		67	98	76	185	168
Total in 60 min.....		230		333		327	370	351	675	710

	0.04 cc. glucose		0.4 cc. glucose		0.4 cc. glucose + 0.4 cc. NaCl		0.8 cc. glucose	
	0.1 cc. malate		0.1 cc. malate		0.1 cc. malate		0.1 cc. malate	
	48	58	74	84	134	176	92	104
First 10 min.....	45	51	49	57	76	169	51	62
Next 10 "	142	165	176	200	268	530	204	243
Total in 40 min.....								
	Phosphate		NaCl, 0.6 cc.		Sulfate			
	0.6 cc.		1.0 cc.		0.3 cc.		0.6 cc.	
	0.3 cc.	0.1 cc. malate + 0.3 cc. NaCl	0.1 cc. malate	0.1 cc. malate	0.1 cc. malate	0.1 cc. malate + 0.3 cc. NaCl	0.1 cc. malate	1.0 cc.
No addition								
First 10 min.....	45	44	40	173	59	57		50
Next 10 "	44	39	35	252	54	57		54
Total in 60 min.....	171	139	123	846*	195	211		209
First 10 min.....		40	42	38	145	180	64	61
Next 10 "		43	47	48	135	184	59	65
Total in 100 min.....		185	195	186	630	1193	263	332
	No addition		0.6 cc. NaCl		0.6 cc. NaBr		0.6 cc. NaNO ₃	
	0.1 cc. malate		0.1 cc. malate		0.1 cc. malate		0.1 cc. malate	
	49	45	144	210	120	192	40	202
First 10 min.....	42	43	137	208	90	184	49	204
Next 10 "	209	204	785	1293	363	933	171	889
Total in 90 min.....								
First 10 min.....			134	196	123	184	38	164
Next 10 "			140	204	123	198	38	122
Total in 75 min.....			645	1122	433	955	157	443

TABLE I—Concluded

	No addition			0.3 cc. NaCl			0.6 cc. NaCl			NaCl		Na ₂ SO ₄	
	0.4 cc. ma-late	0.8 cc. ma-late	1.2 cc. ma-late	0.1 cc. ma-late	0.4 cc. ma-late	0.8 cc. ma-late	0.1 cc. ma-late	0.4 cc. ma-late	0.8 cc. ma-late	1.0 cc.	0.6 cc.	1.0 cc.	0.6 cc.
First 10 min.....	53	81	173										
Next 10 "	40	58	167										
Total in 80 min.....	228	276	764							114	166	54	65
First 10 min.....				78	137	167				113	188	56	60
Next 10 "				53	73	133				459	1229*	282	318
Total in 125 min.....				268	404	609							

* Rate not yet fallen off.

tonic glucose. With optimal NaCl concentration (0.6 cc.), the initial effect was the same whether 0.1 cc. or 0.4 cc. of malate was added (giving 0.007 and 0.028 *M*) but with the higher concentration of malate the high rate lasted longer. The effect was strongly depressed with 0.8 cc. of malate. The results were similar with less chloride present (0.3 cc.) except that in all cases the respiration fell off sooner. Evidently the inhibition due to high malate is not caused purely by high osmotic pressure, since the same effect is seen with the lower salt concentration.

Malate itself can replace chloride to some extent. In the absence of added salt the respiration could be increased by the addition of large amounts of malate; 0.4 cc. of malate gave a brief effect, 0.8 cc. a longer effect, but with 1.2 cc. it fell off again. In no case did the effect last as long as with 0.4 cc. of malate + 0.6 cc. of NaCl. With 0.6 cc. of glucose present, though adding 0.1 cc. of malate caused no increase, 0.4 cc. of malate produced the full effect. These effects may be due to univalent anion provided by malate itself, since a small amount of singly dissociated malate would be present at the pH of the experiments.

The same influence of chloride and a smaller, definite increase in respiration by 0.025 *M* malate in isotonic glucose were found in bicarbonate-buffered medium in an atmosphere of 5 per cent CO₂ in oxygen (Table II). Table II also gives results showing the inhibitory effect of traces of Ca.⁴ For these experiments 1.33 gm.

⁴ An experiment was carried out in the Dixon-Keilin apparatus with sliced liver in bicarbonate-NaCl medium free of Ca, with and without added malate. The results were exactly like those previously obtained (5) in Krebs' medium containing Ca. The lack of effect of added malate on metabolism of liver slices is, therefore, not due to Ca inhibition. Since the respiration of suspensions, even with malate, falls off early, it seemed possible that the time taken to prepare the slices and the rather long standing in the aerated medium before the start of the experiments in the Dixon-Keilin apparatus might result in loss of coenzymes necessary for malate oxidation. Slices were therefore prepared rapidly from the same liver as in the above experiment, not rinsed, but weighed and dropped directly into Ca-free medium in the vessels. The gas was passed faster than usual but for a shorter period. The respiration was somewhat higher than previously observed but was not increased by malate. (Usual method, $-QO_2 = 10.5$ with malate, 10.8 without; rapid method, $-QO_2 = 15.1$ with malate, 16.2 without.) Malate raised the R.Q. from 0.8 to 0.9 and decreased the Q_{acid} from +3.2 to -0.5, small changes indicating slight malate oxidation under the improved conditions.

of tissue were homogenized in 0.025 M bicarbonate with or without other salts and made up to 14 cc. with the medium. 3 cc. of the

TABLE II

Effects of Salts on Respiration of Liver Suspensions in Bicarbonate and in Phosphate Buffer, with and without Added Malate

286 mg. of moist tissue in 3 cc. of medium.

The figures give total gas exchange in c.mm. during 90 minutes.

Medium	Malate	O ₂	CO ₂	R.Q.	Acid change
	M				
0.017 M phosphate + 0.084 M NaCl		164			
	0.007	740			
	Extra*	576			
0.025 " bicarbonate + 0.84 " "		169	100	0.59	+52
	0.007	660	463	0.70	+18
	Extra	491	363	0.74	-34
0.025 " " + no NaCl		125	60	0.5	+72
	0.007	176	87	0.5	+77
	Extra	51	27		+5
0.017 " phosphate + 0.14 M NaCl		163			
0.017 " " + 0.084 " "	0.025	844			
	Extra	681			
0.025 " bicarbonate + 0.12 " "		233	135	0.58	+114
	0.025	1114	923	0.83	-159
	Extra	881	788	0.89	-273
0.025 " " + 0.12 " " + 0.0027 M Ca		113	57	0.5	+57
	0.025	122	53	0.4	+44
	Extra	9			-13
0.017 M phosphate + 0.14 M NaCl		219			
0.017 " " + 0.084 " "	0.025	1148			
0.025 " bicarbonate + 0.22 " glucose		202	94	0.5	+73
	0.025	403	318	0.8	+48
Krebs' complete physiological medium† (containing Ca)		96	46	0.5	+68
	0.025	120	59	0.5	+63

* Due to malate.

† Containing bicarbonate 0.025 M, NaCl 0.12 M, Ca 0.0025 M, and traces of KCl, phosphate, sulfate, Mg, and 0.02 per cent glucose (23).

suspension were taken and the experiment was done in the Dixon-Keilin apparatus. With this apparatus, the suspension has to be kept nearly 20 minutes at 38° during the gassing and equilibra-

than without addition. The rate with oxaloacetate fell off earlier than with the other substances. Acetate and tartrate were without effect. All these active substances induced about the same initial rate of oxygen uptake and there was no sign of an additive effect when two substances were added together. Their action, except in the case of lactate, was completely dependent on the presence of NaCl (presumably univalent anion). With lactate a definite extra oxygen uptake occurred even without added NaCl; with NaCl the rate was increased. The concentration of the lactate added was too low for it to exert appreciable univalent anion effect itself. It seems probable that lactate is oxidized to pyruvate and that the mechanisms for this step do not require the presence of univalent anion for their activity.

Respiratory Quotients—Stare and Baumann (10) and Banga (24) showed that the addition of small amounts of fumarate to minced muscle caused increased respiration with an R.Q. of near unity, which indicated that the fumarate was catalyzing the oxidation of normal, mostly carbohydrate, substances. The same was shown by Greville (19) with fine dispersions of pigeon muscle. With larger amounts of fumarate, 0.02 to 0.026 M, the R.Q. was raised to 1.20 to 1.26 (10), indicating that fumarate itself was being oxidized to some extent. (The R.Q. for complete oxidation of fumarate or malate is 1.33.)

In Tables II and IV the effects of malate on the R.Q. of homogenized liver are shown. With the simple Barcroft method mentioned above,² it was possible to measure the R.Q. during the initial period when the residual respiration was still quite rapid, and during the next period when the residual respiration had largely ceased and nearly all the respiration in the presence of malate was due to the malate. It is seen (Table IV) that with normal liver the R.Q. in either case was less than unity, showing that scarcely any fumarate was being oxidized away, most of the respiration being due to normal substrates. With livers of fasted animals the respiration with malate was as high as usual, but the R.Q. was low, about 0.7, indicating that, in the absence of glycogen, malate can bring about the oxidation of substances other than carbohydrate.

In bicarbonate buffer also, see Table II, the R.Q. with added malate was well below unity. The use of the Dixon-Keilin appara-

tus disclosed a removal of acid groups, especially when a fairly high (0.025 M) malate concentration was used. This probably indicates some complete oxidation of malate, but the acid group

TABLE IV

Effects of Added Malate, Pyruvate, and Citrate, on O₂ Uptake and Respiratory Quotient of Liver Suspensions

286 mg. of moist tissue in 3 cc. of 0.012 M phosphate buffer, pH 7.4. Added NaCl, 0.084 M, in all.

	Time	O ₂	CO ₂	R.Q.
Fed rats				
	min.	c.mm.	c.mm.	
No addition	60	400	232	0.58
Malate, 0.028 M	60	1161	1095	0.94
No addition	First 35	172	134	0.78
	Next 45	61	55±	
Malate, 0.028 M	First 35	606	532	0.88
	Next 45	483	449	0.93
Rat fasted 29 hrs.				
No addition	First 25	252	175	0.69
	Next 35	75	15±	
Malate, 0.007 M	First 25	583	418	0.72
	Next 35	382	239	0.63
Fed rats				
No addition	First 30	367	253	0.69
	Next 50	136	22±	
Pyruvate, 0.014 M	First 30	574	640	1.11
	Next 50	584	513	0.88
No addition	First 25	291	198	0.68
	Next 25	85	62	
Citrate, 0.007 M	First 25	611	633	1.04
	Next 25	430	453	1.05

removal was not nearly sufficient to account for the whole extra oxygen uptake as malate oxidation. For instance, in the second experiment of Table II, the acid disappearance corresponded to 273 c.mm. which, if entirely due to malate removal,

would account for 410 c.mm. of O_2 , whereas actually 881 c.mm. of extra O_2 were taken up. In other experiments with 0.025 M malate, less acid disappeared, and in low malate concentration, 0.007 M, the acid disappearance was negligible and the malate effect appeared to be almost entirely catalytic.

With pyruvate, the R.Q. was raised considerably; during the initial period it was greater than unity and the R.Q. of the extra respiration was high. Evidently this substance does not behave purely as a catalyst but is oxidized itself considerably. (The R.Q. for complete oxidation of pyruvate is 1.2; see also next section.) In one experiment with citrate the R.Q. was raised slightly above unity.

Formation and Removal of β -Ketonic Acids—The formation of acetoacetate from pyruvate by liver has been shown by various workers (25-29). By qualitative nitroprusside tests, Annau (30) showed that small amounts of acetone body were formed by liver mince without addition; with added pyruvate considerably more was formed. While the inhibitor, malonate, increased the accumulation of acetone bodies, the addition of fumarate diminished it. He concluded that fumarate catalyzed the normal oxidation of pyruvate, and in its absence acetoacetate was formed by a less normal condensation and oxidation. It seems equally possible that fumarate catalyzes the oxidation of the acetoacetate. At the end of a number of experiments β -ketonic acids were estimated by the aniline citrate method (31, 27);⁵ the results shown in Table

⁵ For the estimation of the β -ketonic acids at the end of a respiration experiment, 0.3 cc. of 3 N HCl was added to the tissue suspension in the manometer vessel; the alkali paper was removed and alkali carefully washed out with acid, a medicine dropper being used. A Keilin hanging tube containing 0.3 cc. of aniline citrate was introduced. The left, control, vessel containing 3 cc. of water also received HCl and a hanging tube of aniline citrate. The vessels were attached to the manometer again and returned to the bath at 37° (a cool bath is better but was not conveniently available). After 10 minutes shaking the taps were closed. Readings were taken for the next 25 minutes, slight CO_2 evolution occurring, and, by extrapolation, the zero reading at the time of introduction of the vessels into the bath was estimated. The tubes of aniline citrate were then tapped in. After 10 to 15 minutes the CO_2 evolution was complete. (A very slow gas absorption may occur later.) The method under these conditions does not differentiate between oxaloacetic and acetoacetic acids. 1 molecule of either yields 1 molecule of CO_2 .

V confirm the observations of Annau. It is seen that the equivalent of 19 to 50 c.mm. of acetoacetate was formed without addition; with added pyruvate 100 to 140 c.mm. were formed. A considerable amount was formed from lactate and an appreciable

TABLE V
Formation and Disappearance of β -Ketonic Acids (Acetoacetic and Oxaloacetic)

286 mg. of moist tissue in 3 cc. of 0.012 M phosphate buffer. NaCl added, 0.084 M, to all except last three of Experiment 1. All substrates 0.007 M except in Experiments 5 and 6.

The figures represent c.mm. of CO₂ liberated by aniline.

Experiment No.	β -Keto acid after	No addition	Malate	Pyruvate	Lactate	Citrate	No NaCl		
							No addition	Pyruvate	Lactate
1	min.								
2	70	19	11	142	78	37	11	12	13
	75	27				50			
					Oxaloacetate*	Oxaloacetate + pyruvate	Malate + pyruvate	Oxaloacetate + malate	
3	40	25	6	107	20	47	37	5	
4	10	12	7	31					
	40	47	0	105					
	105		0	137					
			Malate, 0.028 M	Oxaloacetate, 0.028 M	Oxaloacetate, 0.028 M in buffer + NaCl; no tissue				
5	0			1380	25 (evolved spontaneously, 10 min.) 46 (" " " 30 ")				
	11			417					
	30			15					
	140	19	22	32					
6	90	24, 25	40, 36						

* Oxaloacetate, estimated in presence of tissue immediately after addition, gave 470 c.mm. of CO₂.

amount from citrate. Added malate or oxaloacetate, 0.007 M, decreased the β -keto acid found, though when a higher concentration, 0.028 M, was used, some oxaloacetate apparently remained (Experiments 5 and 6). Table V, Experiment 4, shows that the acetoacetate was formed rapidly in the first 30 minutes; its rate

of formation then fell off while respiration still proceeded rapidly. It is to be noticed that in the absence of NaCl, lactate and pyruvate do not give acetoacetate. The formation of some acetoacetate may partly account for the raised R.Q. values obtained with added pyruvate or citrate (the R.Q. for oxidation of pyruvate to acetoacetate is 2.0, for citrate it is 4.0).

Stare (20) showed that liver mince caused a very rapid disappearance of added oxaloacetate, and that most of the disappearance could be accounted for by reduction to malate, the remainder by decomposition to pyruvate. The results given in Table V, Experiment 5, also show the rapid removal of oxaloacetate. It should be noted that in spite of the fact that added oxaloacetate was accepting H at an extremely rapid rate, the rate at which oxygen was reduced, that is the oxygen uptake, was not decreased.

Variations in Activity of Livers—Occasionally livers were obtained (eight out of a total of forty-five livers) which showed no effect of added malate under the usual conditions. Usually the residual oxygen uptake of such livers, in the presence of NaCl, was low. By chance an experiment was done with one of these inactive livers in which the amount of tissue was varied. The results are shown in Fig. 1, *B*. It is seen that with a higher concentration of liver the effect of malate became apparent. An experiment with a liver which was active in the usual conditions showed that with a lower concentration of tissue the malate effect was lost (Fig. 1, *A*). When there was no malate effect, the respiration, with or without malate, fell off from the start instead of being constant at a high rate for an initial period. It seems possible that some component of the system has to be present in a minimum concentration in the medium for the respiration to be maintained and the malate to have an effect. Certain livers are comparatively deficient in this component. Possibly in all livers the component is destroyed by oxidizing conditions so that respiration falls off after a time, though added malate, by maintaining connection with the reducing donators, is usually able to preserve the component for a longer time.

Bearing on this point is the fact that homogenized liver loses its activity quite rapidly on standing, even in the refrigerator. But a liver may be kept on ice for several hours before being homog-

enized and still show almost its full activity. Presumably the intact tissue keeps the component in a reduced, stable, condition.

The reason for the occurrence of the "inactive" livers is obscure. On several occasions when two rats in the same apparently good condition were taken from the same cage, the liver of one was "inactive" and the other fully active under identical experimental conditions. That the variation does not depend on the glycogen content of the liver was shown by several experiments with livers

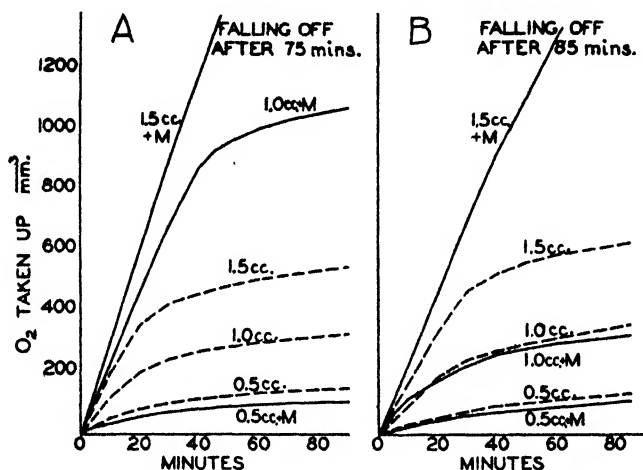


FIG. 1. Effects of varying concentrations of liver tissue (A, "active;" B, "inactive") on the oxygen uptake with and without added malate. The vessels received 1.5, 1.0, or 0.5 cc. of liver suspension, in 0.05 M phosphate buffer, equivalent to 429, 286, or 183 mg. of moist tissue. With 0.5 cc. of suspension, 0.5 cc. of extra 0.05 M buffer was added. For curves marked M, the vessels received 0.4 cc. of 0.21 M *l*-malate solution plus 0.6 cc. of 0.42 M NaCl solution. Others received 1.0 cc. of NaCl solution. All volumes were made to 3 cc. with water.

of rats fasted 24 to 30 hours. These livers were always remarkably active.

Other Tissues—Work has been done on homogenized brain, kidney, and testis. Brain and kidney behave very similarly to liver with certain differences. The behavior of testis suspension is entirely different (possibly owing to incomplete rupture of sperm cells). These results will be reported later.

DISCUSSION

There are two theories concerning the catalytic action in animal tissues of the metabolites studied in this paper. According to von Szent-Györgyi and his school (8), succinate-fumarate and malate-oxaloacetate with their dehydrogenases serve as hydrogen transporters between donators and the oxygen-activating system. According to Krebs (4, 9) these substances, with citrate and α -ketoglutarate, represent steps in a cycle of reactions which includes a condensation between oxaloacetate and "triose," or other carbohydrate derivatives, which yields citrate. Krebs' theory has been critically examined by Breusch (32) and the question is not settled. It is possible that citrate and α -ketoglutarate are oxidized and that the fumarate, etc., formed, act as catalysts in the von Szent-Györgyi sense. This might also be the manner in which pyruvate, which Krebs does not include in his cycle, exerts most of its effect.

A point to be noted is that with all these substances, including pyruvate and oxaloacetate, the initial oxygen uptake rate is about the same and the effects of the different substances are not additive (Table III). Also the effects of all of them are dependent on the presence of univalent anion. Oxaloacetate when first added acts as a hydrogen acceptor at a very high rate, corresponding to an oxygen usage of 1200 to 2700 c.mm. of O_2 per hour under the conditions of these experiments, yet the actual initial oxygen uptake is still as rapid as with the other substances added. It therefore appears that in all cases the rate of oxygen uptake is limited by the same process, probably the process which requires the presence of univalent anion. The discovery of what this limiting process is should help in the elucidation of the whole respiratory process. Banga (16), Stare and Baumann (10), Greville (19), and Krebs and Eggleston (9) have shown that heated muscle extracts increase the respiration of muscle suspensions with fumarate. These increases are possibly partly due to NaCl in the extracts but probably mainly due to the presence of a coenzyme which is concerned in the limiting process. Krebs and Eggleston (9) have shown that a further marked increase in the respiration of muscle mince is produced by small additions of insulin. Insulin, therefore, may be concerned in the limiting process.

Banga (16) mentioned that in Ringer's solution the reduction of

oxaloacetate to malate by muscle suspension was inhibited. This was probably due to the Ca in her Ringer's solution and her observation may help fix the point of action of Ca.

SUMMARY

1. The rate of respiration of liver suspensions is increased in the presence of chloride or other univalent anions. The respiration can also be increased for a short time by raising the osmotic pressure with glucose. With glucose a higher osmotic pressure is required than with NaCl.

2. The respiration is further increased, and maintained at a high level for a longer period, by the addition of malate, oxaloacetate, citrate, α -ketoglutarate, and pyruvate. The action of these substances is completely dependent on the presence of chloride (univalent anion). Lactate also increases the respiration but part of the increase, presumably that due to oxidation to pyruvate, is not dependent on chloride.

3. Measurements of R.Q. with livers of normal and fasted rats show that the effect of added malate is largely catalytic and that non-carbohydrate materials as well as carbohydrate derivatives are probably burnt as a result of this catalysis. Some malate is completely oxidized, at least in bicarbonate buffer. Pyruvate and, to a lesser extent, citrate raise the R.Q., and this may be due to the fact that a considerable amount of acetoacetate is formed from pyruvate and some from citrate (the R.Q. for conversion of pyruvate to acetoacetate is 2.0, for citrate it is 4.0).

4. Some β -ketonic acid (acetoacetic) is formed by the respiring tissue, considerably more when pyruvate is added. The amounts are decreased by the presence of added malate or oxaloacetate. The formation of acetoacetate from pyruvate does not occur in the absence of NaCl.

5. Added oxaloacetate is removed, mostly by reduction to malate (20); *i.e.*, it acts as H acceptor, at a very rapid rate, even in the presence of oxygen, yet the initial rate of oxygen uptake is the same as with added malate.

6. The effect of malate is dependent on a certain minimum concentration of tissue. Occasionally livers are obtained with which this limiting concentration is unusually high. The responsiveness to malate keeps for several hours in intact excised liver but is soon lost after dispersion in buffer, even in the cold.

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THE SYNTHESIS OF DICHOLYLCYSTINE AND CHOLYLCYSTEIC ACID*

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The mechanism by which taurocholic acid is formed in the body has not yet been established. It has been suggested that the taurine is derived from cystine by the series of reactions, cystine→cysteic acid→taurine, which has long been known to the organic chemist (1, 2). The possibility that taurine, thus formed, is conjugated directly with cholic acid is suggested by experiments in which simultaneous feeding of taurine and cholic acid to dogs increased the alcohol-soluble sulfur of the bile, whereas either taurine or cholic acid fed separately under suitable conditions did not alter the amount of this sulfur fraction or of the conjugated amino nitrogen in the bile (3, 4). It has also been observed that the alcohol-soluble sulfur of the bile increases after the administration of cystine and cholic acid together (3, 4).

If, in the living organism, taurine is derived from cystine, it is possible that the conversion of cystine to taurine may occur before the conjugation of the latter substance with cholic acid. The behavior of taurine when fed with cholic acid favors this mechanism. However, it is also possible that cystine is conjugated with cholic acid and the cholylcystine thus formed is converted to taurocholic acid (cholyltaurine). Such a mechanism is supported by the experiments of Blum (5) who injected cystine into the vein of a dog and was able to obtain from the bile an alcohol-soluble substance containing labile sulfur and cholic

* A preliminary report of this investigation was presented before the Thirty-second annual meeting of the American Society of Biological Chemists at Baltimore, March 30–April 2, 1938 (Velick, S. F., and White, J., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **123**, p. cxxiii (1938)).

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acid. These properties suggest the presence of dicholylcystine or some similar substance in the bile.

The following series of reactions may represent the biological synthesis of taurocholic acid (cholyltaurine): cholic acid + cystine \rightarrow dicholylcystine; dicholylcystine \rightarrow cholylcysteic acid; cholylcysteic acid \rightarrow cholyltaurine.

In order to study this proposed mechanism, dicholylcystine¹ and cholylcysteic acid have been synthesized. Triformylcholic acid was converted to the acid chloride and coupled in chloroform solution with the dimethyl ester of cystine. Selective hydrolysis in dioxane converted the ester to the acid. The formyl groups were removed by the action of anhydrous ammonia in methyl alcohol and the product was isolated as the diammonium salt. Acidification with dilute hydrochloric acid produced the free acid. Cholylcysteic acid was prepared by the oxidation of di(tri-formylcholyl)-cystine dimethyl ester with bromine and subsequent removal of the formyl groups with sodium methylate. The cholylcysteic acid was isolated as the sodium salt.

EXPERIMENTAL

Triformylcholic Acid—This compound was prepared by a method essentially the same as that of Cortese and Baumann (7). 50 gm. of cholic acid (Hoffmann-La Roche), m.p. 196°, were dissolved in 100 cc. of redistilled formic acid and the mixture was heated for 6 hours at 60° in a glass-stoppered flask. The clear yellowish reaction mixture was poured slowly and with vigorous stirring into several volumes of distilled water containing cracked ice. The white granular precipitate which formed was filtered by suction, dissolved in 500 cc. of hot 95 per cent alcohol, and 600 cc. of boiling water were added gradually. After standing 5 hours at room temperature, the crystals were filtered off and recrystallized again. 50 gm. were obtained. Yield, 83 per cent. M.p. 206°.

¹ After the completion of this work, the synthesis of cystocholic acid (dicholylcystine) was reported from Sobotka's laboratory by Holzmann (6). Beyond the statement that the synthesis was carried out by the procedure of Curtius, no details are available. We also prepared small amounts of dicholylcystine by the azide method, but abandoned this method, since better yields were obtained by the more convenient procedure described in this paper.

Triformylcholy Chloride—The method of Cortese and Baumann (8) was employed. It was necessary to distil the thionyl chloride from quinoline and then from linseed oil in a moisture-free chamber immediately before each run. Unless these precautions were taken, the product became highly colored.

Dimethyl Ester of Di(Triformylcholy)-Cystine—A solution of 10 gm. of freshly prepared triformylcholy chloride dissolved in 100 cc. of dry chloroform was added gradually to twice the equivalent amount of the dimethyl ester of cystine in 70 cc. of chloroform. The solution was allowed to stand for 3 hours at room temperature. The precipitated ester hydrochloride of cystine was removed by filtration and the filtrate was extracted several times with 10 per cent hydrochloric acid, then with 5 per cent sodium carbonate, and finally with water. The chloroform solution, after extraction, was dried over sodium sulfate and the chloroform was removed *in vacuo* on a water bath at 60°. The residue was crystallized from hot capryl alcohol. The needles melted at 88–90°. Yield, 70 per cent.

$C_{62}H_{84}O_{12}N_2S_2$. Calculated, N 2.29, S 5.25; found, N 2.16, S 5.08

Diammonium Salt of Dicholylcystine—The dimethyl ester of di(triformylcholy)-cystine was dissolved in 10 times its weight of dioxane, 8 equivalents of *N* sodium hydroxide were added, and the solution was shaken in a stoppered flask for 20 minutes. An amount of *N* hydrochloric acid equivalent to the alkali used was added and the dioxane was distilled off *in vacuo*. This procedure removed the methyl groups and some of the protecting formyl groups. Without further purification, 2 gm. of the dried product were dissolved in 20 cc. of dry ethyl alcohol that had been saturated with anhydrous ammonia and the solution was allowed to stand 16 hours at room temperature in a glass-stoppered flask. The ammonia and the alcohol were then distilled off *in vacuo*. The residue was extracted with ether and dried *in vacuo* over phosphorus pentoxide. Yield, 87 per cent.

$C_{34}H_{44}O_{12}N_4S_2$. Calculated, N 5.31, S 6.07; found, N 5.09, S 6.13

Dicholylcystine—The clear colorless water solution of the above diammonium salt was precipitated with 10 per cent hydrochloric acid. The white, powdery precipitate was filtered by suction,

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washed with water, and dried *in vacuo* over phosphorus pentoxide. The yield was almost quantitative.

$C_{44}H_{88}O_{12}N_2S_2$. Calculated, N 2.74, S 6.26; found, N 2.81, S 6.17

Methyl Ester of Triformylcholylcysteic Acid—2 gm. of the dimethyl ester of di(triformylcholyl)-cystine were pulverized to a fine powder and suspended in 50 cc. of water, and the mixture was stirred mechanically. 1.29 gm. of bromine were added in small portions as rapidly as the color of the bromine disappeared. The solution was filtered from a trace of unoxidized ester, extracted with ether, and shaken with a slight excess of moist silver oxide. The solution was filtered rapidly and the filtrate was treated with hydrogen sulfide to remove the excess silver. The silver sulfide could not be readily filtered off, as it was present in colloidal form. However, by concentrating this colloidal solution to dryness *in vacuo* on a water bath, and by subsequently extracting with dry methyl alcohol, a clear solution free from silver was obtained. The straw-colored extracts were decolorized with carbex E and the alcohol was removed in a vacuum desiccator over sulfuric acid. The colorless product was dried *in vacuo*. Yield, 77 per cent.

$C_{31}H_{47}O_{12}NS$. Calculated, N 2.13, S 4.87; found, N 2.10, S 4.90

It is not necessary to isolate this compound for the succeeding steps inasmuch as the dry methyl alcohol solution can be used directly.

Ammonium 26-Carbamyltaurocholate (β -Carbamyltaurocholate)—A solution of 1 gm. of triformylcholylcysteic acid (methyl ester) in 20 cc. of anhydrous methyl alcohol was saturated with dry ammonia and was allowed to stand for 16 hours at room temperature. The alcohol and excess ammonia were removed *in vacuo*. The residue was dissolved in methyl alcohol, decolorized by boiling with a little carbex E, filtered, and 3 volumes of dry ether were added. The precipitate was filtered off and dried in a vacuum desiccator.

$C_{27}H_{43}O_6N_2S$. Calculated, N 7.30, S 5.55; found, N 7.39, S 5.52

This derivative was prepared as a confirmatory compound for analysis.

Disodium Salt of Cholylcysteic Acid—1.55 gm. of the methyl ester of triformylcholylcysteic acid (from 2 gm. of the dimethyl ester of di(triformylcholyl)-cystine) were dissolved in 10 cc. of dry methyl alcohol and a solution of 0.72 gm. of sodium in 10 cc. of dry methyl alcohol was added. After standing at room temperature for an hour, the finely divided white precipitate was filtered off and washed with dry alcohol and ether. It was then dissolved in a hot solution of 5 cc. of absolute methyl alcohol and 2 cc. of water. 8 cc. of absolute methyl alcohol were added and the solution was chilled overnight. The supernatant liquid was decanted from the colorless oil which separated. The oil was washed with dry alcohol, was covered with dry ether, and was allowed to stand 6 hours. The solid mass was pulverized, filtered, and dried *in vacuo*.

$C_{27}H_{45}O_5NSNa_2$. Calculated, N 2.32, Na 7.6; found, N 2.25, Na 7.3

The biological studies of these compounds will be reported at a later date.

SUMMARY

The peptide, dicholylcystine, has been synthesized and its oxidation to cholylcysteic acid is described. These compounds are of interest in view of the suggestion that the conjugation of cholic acid with cystine may occur *in vivo* as a reaction in the biological synthesis of taurocholic acid (5, 9).

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NOTE ON TESTS FOR PURITY OF SOLID SILVER IODATE PREPARED FOR CHLORIDE DETERMINATION

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The accuracy of the writer's chloride method¹ depends on the use of pure silver iodate. The preparation and suggestions for the testing of the AgIO_3 were given in foot-note 2 on p. 338 of the original paper; nevertheless, in one of the laboratories in which the method has been used it was found that too high results may be obtained because of the presence of unnoted traces of KIO_3 in the AgIO_3 . For this reason it seems desirable to state more fully the following points with regard to the preparation and testing of the AgIO_3 .

1. When the AgIO_3 is made by precipitation of AgNO_3 solution with KIO_3 , the directions given in the original paper should be followed precisely. In particular, although an excess of KIO_3 is used, that excess should be only *slight*; a larger excess than that prescribed may make difficult the removal of KIO_3 from the AgIO_3 by washing.

2. Before use, every preparation of AgIO_3 , whether made in the laboratory, or purchased commercially, should be tested quantitatively for the presence of KIO_3 . The test can be made in either of two ways. The solubility of the AgIO_3 in water can be measured, or the AgIO_3 can be used in analyses of standard chloride solutions of exactly known Cl content. It is in fact well to use both tests when introducing the method.

Solubility Measurement—This is made by the technique described on p. 368 of the original paper, with the gasometric, titrimetric, or colorimetric determination of the IO_3 in the saturated

¹ Sendroy, J., Jr., *J. Biol. Chem.*, **120**, 335, 405, 419 (1937).

water. The IO_3 in the supernatant water *should not exceed 0.21 mm per liter.*

In the *gasometric* analysis the procedure described on p. 368 is to be followed,² with the additional precaution that, after the solution has been extracted for 3 minutes and the p_1 reading has been taken, the completeness of the extraction is checked by again lowering the mercury in the chamber to the 50 cc. mark, shaking the chamber for 1 minute, and repeating the p_1 reading. If any increase over the first reading is noted, the 1 minute extraction and p_1 reading are repeated, until the p_1 is constant. Because of the unusually large volume of solution (10 cc.) in the chamber, and the extreme dilution of the iodate solution, the reaction and extraction are not so quick as under the conditions of the actual chloride determinations. If the AgIO_3 is pure, the IO_3 in solution will give a $p_1 - p_0$ difference of about 54 mm. at 0.5 cc. volume (the p_0 reading being that of the blank analysis in which water replaces the supernatant solution). The results are calculated according to Equation 4 (p. 362), with the $F (= f/3)$ factor taken from Table III (p. 357).

In the *titration* (p. 368), if 3 cc. of supernatant solution are titrated, approximately 3 cc. of 0.0012 N thiosulfate solution should be required, according to Equation 3 (p. 411). Thus,

$$\text{mm IO}_3 \text{ per liter} = \frac{3 \times 0.0012 \times 1 \times 1000}{6 \times 1 \times 3} = 0.2$$

For the slight amounts of iodate measured, the titration will usually be found more convenient and reliable than the gasometric measurement. The colorimetric measurement (p. 422 and Table I, p. 424) is also convenient.

² If a 5 cc. stop-cock pipette is lacking, a 5 cc. bulb pipette may be used. The hydrazine is first admitted into the chamber, which is then sealed with mercury. The cup is then washed with water several times. After all excess mercury and water have been removed, the sample is admitted into the cup, and simultaneously into the chamber. Traces of sample adhering to the wall of the cup are then diluted with water up to the 2 cc. mark on the cup. These additional 2 cc. are admitted into the chamber, which is again sealed, and evacuated as usual. The blank analysis with water must be carried out with the same type of pipette and technique used for the iodate analysis.

Analysis of Standard Chloride Solution—This is carried out as described on pp. 346 and 370 of the original paper. The simplest procedure is to analyze a known 100 mM chloride solution diluted 20 times with 0.085 M H_3PO_4 . For the gasometric analysis, 1 cc. samples of supernatant are used, and readings are made at the 0.5 cc. volume. Results are calculated according to Equation 5 (p. 363). For the titrimetric analysis, 3 cc. samples of supernatant are used, and titrated with 0.03 N $\text{Na}_2\text{S}_2\text{O}_3$. Results are calculated according to Equation 4 (p. 414).

3. An additional precaution may be worth noting in connection with the use of a cotton plug (p. 342) for filtering the sample into a pipette. If the plug is inserted too tightly, and too much suction is exerted, the air content of 2 cc. of pipetted solution may be diminished enough to lower by as much as 2 mm. the pressure of the extracted gas when the pressure is measured at 2 cc. volume. In analysis of a 1:10 serum filtrate this maximal error would decrease the gasometrically determined serum Cl by 0.7 mM per liter. This error, which, of course, does not occur in the titration or colorimetric measurement, would ordinarily not be important, but it is easily avoided.

THE METABOLISM OF N-METHYLATED AMINO ACIDS

I. THE AVAILABILITY OF α -N-MONOMETHYLLYSINE AND α -N-DIMETHYLLYSINE FOR GROWTH*

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(Received for publication, November 21, 1938)

In 1935 Gordon and Jackson observed that young rats can metabolize N-monomethyltryptophane in place of tryptophane for purposes of growth. Of possible mechanisms for conversion of the N-methyl derivative to the amino acid the most likely was postulated to be a demethylation with the production of indolelactic acid or indolepyruvic acid, substances which are known to be capable of replacing tryptophane for the promotion of growth. The suggestion was made at the same time that other N-methylamino acids might be utilized in metabolism in place of the corresponding natural unsubstituted acids.

Subsequently, growth studies by Fishman and White (1936) proved that amino-N-monomethylhistidine can function as a substitute for histidine and similar experiments by Patterson, Dyer, and du Vigneaud (1936) demonstrated that the N-monomethyl derivatives of homocystine and methionine are able to support the growth of rats maintained on a cystine-methionine-deficient diet. In these studies too, the most plausible mechanism for the conversion of the methyl derivatives to their respective amino acids was considered to be an intermediary demethylation to the corresponding α -ketonic acids.

Experiments with a number of N-monomethylated amino acids both in model systems and with amino acid deaminase also have indicated that these compounds are oxidized with the production

* Aided by a grant from the Stanford University School of Medicine Research Fund. A preliminary report of this work was presented before the meeting of the American Society of Biological Chemists at Memphis, April, 1937.

of methylamine and the corresponding α -ketonic acids (Krebs, 1936).

In view of these findings it was thought of interest to investigate the availability of α -N-monomethyllysine to the animal organism as a substitute for lysine. It will be recalled that McGinty, Lewis, and Marvel (1924-25) observed that the rat cannot utilize α -hydroxy- ϵ -aminocaproic acid in place of lysine for purposes of growth. The corresponding α -ketonic acid has not been synthesized.

The study of the behavior of α -N-dimethyllysine as a substitute for lysine was undertaken, since experiments *in vitro* have shown that at least 1 free hydrogen atom must be attached to the amino nitrogen atom of amino acids if the process of oxidative deamination is to occur (Krebs, 1936). There have been but few *in vivo* investigations concerning the metabolism of N-dimethylamino acids. The data obtained by Friedmann in 1908 led him to the conclusion that N-dimethylamino acids could be catabolized as well as N-monomethylamino acids. However, as Knoop and Oesterlin (1927) have pointed out, the criterion for catabolism in Friedmann's experiments was a comparison of urinary carbon to nitrogen ratios and for this reason the validity of his conclusion is subject to serious doubt. Knoop and Oesterlin, on the basis of the results of their own studies with N-dimethylphenylalanine, were of the opinion that N-dimethylamino acids could not be metabolized by the animal organism.

The present experiments with α -N-dimethyllysine were expected to provide additional information regarding the behavior of N-dimethylamino acids in metabolism.

EXPERIMENTAL

Preparation of Methyllysines

α -N-Monomethyllysine— ϵ -Benzoylamino-caproic acid was brominated in the α position according to the procedure of Eck and Marvel (1934). The α -bromo acid was then converted to α -N-monomethyllysine by a combination of the methods of Enger and Steib (1930) and Abderhalden and Schweitzer (1930-31). The desired compound was isolated in the form of its crystalline monohydrochloride.

Analysis—

$C_7H_{17}O_3N_2Cl$. Calculated. C 42.72, H 8.72, N 14.25, Cl 18.03
Found. " 43.04, " 8.42, " 14.4, " 17.8

α -N-Dimethyllysine—This compound is not described in the literature but it was synthesized readily by a method quite similar to that employed for the preparation of the monomethyllysine. 20 gm. of α -bromo- ϵ -benzoylaminocaproic acid were dissolved in 90 cc. of 33 per cent aqueous dimethylamine. The solution was allowed to stand at room temperature for a period of 6 weeks, although a much shorter reaction time undoubtedly would have sufficed. A small insoluble residue was removed by filtration and the liquid was then concentrated *in vacuo* to a thick syrup. Attempts to crystallize the product, ϵ -benzoyl- α -N-dimethyllysine, from a number of solvents were unsuccessful. Accordingly, 200 cc. of water and 200 gm. of anhydrous barium hydroxide were added to the syrup and the mixture was boiled under a reflux condenser for 18 hours. In this way any free dimethylamine remaining in the syrup was driven off, the dimethylammonium bromide produced in the reaction was decomposed, and the benzoyldimethyllysine was hydrolyzed to yield the desired product and benzoic acid. The mixture was diluted with water and excess sulfuric acid was added in the usual manner to remove barium. Silver oxide served to precipitate bromides; the excess silver was then precipitated in the form of silver sulfide. Benzoic acid was removed from the solution by filtration and ether extraction. The excess sulfuric acid was finally precipitated by exact neutralization with barium hydroxide.

Following the removal of dimethylamine, benzoic acid, and inorganic ions as outlined above, the solution of dimethyllysine was concentrated *in vacuo* to a syrup which again resisted all attempts at crystallization. The syrup was therefore neutralized to litmus with dilute hydrochloric acid and the solution was evaporated *in vacuo* once more. The neutralized syrup was dissolved in 100 cc. of warm 95 per cent ethyl alcohol, the solution was chilled, and anhydrous ether was added very slowly until no further precipitate formed. The dimethyllysine monohydrochloride, at first somewhat gummy, crystallized completely when it was allowed to remain in the refrigerator overnight. The crystalline solid was filtered off and dried in the air. The dried material

weighed 11 gm. but was manifestly impure. It was recrystallized from 110 cc. of hot 86 per cent ethyl alcohol and yielded a first crop of colorless prisms which weighed 7.4 gm. and melted at 136°;¹ a second crop, which weighed 3.0 gm., melted at 130°. The product, when dried at 106° to constant weight, lost 7.7 per cent of its original weight, indicating the presence of 1 molecule of water of crystallization. Therefore, following a second recrystallization from 86 per cent ethyl alcohol, the compound was dried at 106°. The anhydrous purified monohydrochloride sintered at 220° and decomposed at 232–233°. The yield was 9 gm. (76 per cent of the theoretical amount based on the bromo acid). This material was analytically pure dimethyllysine monohydrochloride.

Analysis—

$C_8H_{19}O_2N_2Cl$.	Calculated.	C 45.58,	H 9.09,	N 13.30,	Cl 16.83
	Found.	" 45.52,	" 9.01,	" 13.3,	" 16.66

Physiological Tests

The methyllysine hydrochlorides were assayed in the usual way for their growth-promoting ability as substitutes for lysine. Young albino rats, weighing approximately 60 gm. each, were used as experimental animals. Two series of experiments were carried out. In Series I the rats were fed a lysine-deficient diet which consisted of gliadin 15, corn-starch 38, sucrose 15, salt mixture (Osborne and Mendel, 1919) 4, ground agar 2, and lard 26 per cent. Each animal received daily 100 mg. of cod liver oil and 1 tablet² (100 mg.) of yeast vitamin concentrate (Harris) apart from the remainder of the diet. The animals were maintained on this basal ration for a period of at least 4 weeks. Next, the derivatives to be studied were incorporated in the deficient diet in amounts equivalent, on the basis of molecular weights, to 1 gm. of *dl*-lysine per 100 gm. of the basal food. The compounds were fed for a period of at least 3 weeks. At the end of this time, since the rats did not show positive growth responses, the deficient diet was restored for a period of 2 weeks, and finally *dl*-lysine itself (in the form of the dihydrochloride) was incorporated in the basal

¹ All melting points are corrected.

² Each tablet contained 15 Sherman units of vitamin B₁ and 4 Sherman units of vitamin B₂, vitamin-free starch being used as excipient.

ration to demonstrate that the animals possessed the expected capacity for growth. All diets were fed *ad libitum* and individual food consumption data were recorded. Each compound was tested with three animals and the results were entirely uniform.

In the experiments of Series II several changes were made in the above procedure because of the fact that the animals did not grow very well even during the period when lysine was included in the diet. In the first place, the percentage of gliadin in the deficient ration was raised to 30, the corn-starch being reduced accordingly to 23 per cent (*cf.* Melnick and Cowgill (1937)). Secondly, the vitamin B complex supplement was administered in a different form: 100 mg. of ryzamin-B³ and 100 mg. of liver extract powder (Lilly) dissolved in 2 cc. of water and fed daily to each animal. Finally, since the amino acid supplements were being fed in the forms of their hydrochlorides, it was thought desirable to neutralize the excess acid; therefore, when the deficient diet was supplemented by monomethyllysine or by lysine, the theoretical quantity of sodium bicarbonate required for neutralization of the hydrochloric acid was included in the supplement. Otherwise the studies in Series II were conducted in exactly the same way as were those in Series I.

Much better growth responses were obtained by means of the modified procedure. However, the experiments in Series II served solely to confirm and strengthen the results of the experiments in Series I. Since the results of the tests with monomethyllysine in both series were similar, the growth responses being negligible in all experiments, the dimethyllysine was not assayed in Series II.

DISCUSSION

The results of the physiological tests are summarized in Table I. In Series I Rats 30 and 45 were negative controls (given the basal deficient diet); Rats 31 and 41 were positive controls (given the basal diet + lysine). The monomethyllysine was tested in Rats 33, 40, and 43. During the period of feeding with this compound little or no change in growth rate was observed. The dimethyllysine was administered to Rats 32, 42, and 44. Again, no positive growth response was apparent. It will be noticed that the

³ This preparation was generously supplied by the Burroughs Wellcome and Company Experimental Research Laboratories.

TABLE I
Average Daily Weight Changes and Food Consumption of Rats Fed a Lysine-Deficient Diet and Various Supplements

	Rat No. and sex	Days	Average daily change in weight	gm.	Average daily food consumption	Rat No. and sex	Days	Average daily change in weight	gm.	Average daily food consumption	Rat No. and sex	Days	Average daily change in weight	gm.	Average daily food consumption	Supplements per 100 gm. basal food
Series I*	30 ♀	1-84	-0.05	2.8	45 ♀	1-84	+0.02	3.0								None
	31 ♀	1-28	+0.04	4.1	41 ♂	1-28	-0.04	3.7								"
		29-70	+0.50	3.5		29-52	+0.58	3.0								1.499 dl-lysine 2HCl
	33 ♂	1-28	-0.11	3.6	40 ♀	1-28	-0.07	3.5				1-28	-0.04	3.3		None
		29-50	+0.05	2.1		29-52	0.00	2.1				29-54	-0.04	2.2		1.346 monomethyllysine HCl
		51-64	0.00	2.5		53-66	+0.07	2.5				55-68	+0.21	2.6		None
Series II*		65-89	+0.60	2.8		67-89	+0.44	2.6				69-91	+0.83	3.3		1.499 dl-lysine 2HCl
	32 ♂	1-28	-0.18	3.6	42 ♀	1-28	-0.18	2.9				1-28	-0.07	3.6		None
		29-49	+0.05	2.6		29-54	0.00	2.3				29-54	+0.08	2.6		1.441 dimethyllysine HCl
		50-63	0.00	2.6		55-68	+0.07	2.6				55-68	+0.07	2.9		None
		64-91	+0.57	3.0		69-91	+0.39	2.6				68-88	+0.71	3.3		1.499 dl-lysine 2HCl
	46 ♀	1-81	+0.37	4.5	48 ♀	1-55	+0.33	4.3								None
	47 ♀	1-28	+0.39	4.3	51 ♀	1-28	+0.46	4.0								"
		29-49	+0.05	3.3		29-55	+0.04	2.9								1.346 monomethyllysine HCl + 0.575 NaHCO ₃
		50-63	+0.50	4.4		56-69	+1.00	4.6								None
		64-77	+4.0	7.6		70-83	+3.4	6.9								1.499 dl-lysine 2HCl + 1.15 NaHCO ₃

* The basal diet in Series I contained 15 per cent gliadin; that in Series II contained 30 per cent gliadin.

experimental animals, during the period of feeding with lysine, grew at a rate of approximately 0.5 gm. per day and that the positive control animals grew at a similar rate. Although the differences in growth rates were not as great as they might have been, it is felt that the results are significant and that they indicate that neither of the methyllysines is available to the rat for purposes of growth. This conclusion is supported by the data from Series II. Control Rats 46 and 48, maintained on the modified basal diet which contained twice as much gliadin as the original basal diet, grew slowly at a rate of approximately 0.35 gm. per day. Experimental Rats 47 and 51 grew somewhat more rapidly on the basal ration but, upon incorporation of monomethyllysine in the food, the growth rate was lowered rather than raised; during the final period of lysine feeding these rats grew quite rapidly.

The failure of α -N-monomethyllysine to promote growth is perhaps surprising in view of the utilization of other N-monomethylated amino acids. It is possible, of course, that monomethyllysine cannot be demethylaminated by the rat and that it therefore cannot function as a precursor of lysine. Although there is little *in vivo* evidence favoring this hypothesis, the *in vitro* experiments of Keilin and Hartree (1936) afford it some support. These investigators, while showing that methylamine is liberated from N-monomethylalanine by the action of amino acid deaminase, also demonstrated that N-monomethyltyrosine is not attacked by the enzyme. It would be interesting to study the behavior of monomethyllysine in this reaction; perhaps, like monomethyltyrosine, monomethyllysine is not attacked.

If we assume, on the other hand, that demethylation of the monomethyllysine does occur in the usual manner, the corresponding α -hydroxy or α -ketonic acid would be produced. It has already been established that α -hydroxy- ϵ -aminocaproic acid cannot replace lysine in metabolism. In this respect then, there is a striking similarity in the behavior of α -N-monomethyllysine and α -hydroxy- ϵ -aminocaproic acid. The failure of animals to utilize either of these substances may well be attributed to a failure in the production of the corresponding α -ketonic acid or to a loss of the keto acid by some side reaction after it is formed. In this connection Jackson and Block (1937-38), referring to the results of the present investigation, comment as follows: "Neither the *dl*-

hydroxy nor the *dl*-N-methyl derivative nor the unnatural form of the amino acid is available in place of lysine for growth. This may not mean the failure or absence of three entirely separate physiological mechanisms. On the other hand, it is conceivable that these three substances related to natural lysine are converted to the corresponding α -keto acid, but that the latter, susceptible to side reaction, is diverted from the more usual amination process."

Obviously these experiments throw no light on the general problem of the metabolism of N-dimethylamino acids. Since α -N-monomethyllysine apparently cannot replace lysine in metabolism, there is little reason to expect anything other than a negative result with the dimethyl compound. It will be interesting to study other N-dimethylamino acids in a similar manner.

SUMMARY

A synthesis of α -dimethyllysine monohydrochloride has been described.

Neither α -N-monomethyllysine nor α -N-dimethyllysine is capable of stimulating growth in rats subsisting on a diet deficient in lysine.

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A CHEMICAL REAGENT FOR THIAMINE*

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The rapid advances in the study of the physiological rôle of thiamine (vitamin B₁) make its direct quantitative estimation highly desirable. Few chemical tests have been proposed which offer the prospect of application to solutions or concentrates for this purpose.

Jendrassik (1) while working in our laboratory tried to find a specific chemical reagent which would produce a qualitative color reaction with the antineuritic vitamin. He found that solutions containing this vitamin invariably reduced ferric ferricyanide to form blue-colored solutions, which he assumed owed their color to the presence of Prussian blue (ferric ferrocyanide). Levine (2) made a critical study of Jendrassik's reaction and found that this test was given by ortho- and polyphenols. Nevertheless the use of ferricyanide may have suggested a line of thought to Barger (3), who oxidized the vitamin with alkaline ferricyanide to form a pale yellow-blue fluorescent compound now known as thiochrome. The thiochrome test has been made the basis of a method for the quantitative estimation of thiamine by Jansen (4), who used the fluorometer apparatus of Cohen (5) for measuring the fluorescence of the thiochrome after it had been extracted with isobutyl alcohol. Certain substances interfere with this reaction, since complete recovery of thiamine added to urine cannot be obtained.

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† Eli Lilly Fellow in Biochemistry 1936-37.

The method also involves the use of special apparatus and the results obtained vary as much as 20 per cent from those obtained by the bradycardia method of Birch and Harris (6).

Spruyt (7) proposed the use of phosphotungstic acid to precipitate thiamine from extracts of foodstuffs and estimated the centrifuged precipitate by means of hematocrit tubes. Rosenthaler (8) reported the use of Reinecke's salt as well as potassium tetranitritodiamino cobaltate for precipitating the vitamin. However, these tests are not specific. Naiman (9) reported the use of a solution of bismuth iodide in potassium iodide as a sensitive reagent for the thiazoles. He suggested the possibility of using this reagent as the basis of a gravimetric determination of thiamine. No specificity was claimed and it is well known that the solution reported is classed with the alkaloidal reagents.

A Pauly (10) color reaction for thiamine was first reported by Jansen and Donath (11) who employed diazotized sulfanilic acid as the reagent. Kinnersley and Peters (12) claimed that their yeast fractions did not fractionate with biological activity when they used the Koessler and Hanke modification of this reaction (13). Guha and Drummond (14) also reported that active anti-neuritic fractions obtained from yeast gave feeble Pauly reactions. Odake (15) claimed that his vitamin B₁ crystals gave a slow, pink diazo reaction. Nevertheless, Tschesche (16) and also van Veen (17) were unable to find any activity of their crystalline thiamine preparations when treated with diazotized sulfanilic acid. Van Veen was of the opinion that a reaction should take place and he was at a loss to explain the inactivity of his preparations with the reagents.

Kinnersley and Peters (18) were of the opinion that fine shades of alkalinity influenced the coupling of the vitamin. When the Koessler and Hanke modification of this reaction was used, a yellow coloration was obtained, whereas solutions more strongly alkaline resulted in pink colors which faded gradually and were always tinged with yellow. Although the formaldehyde modification of Kinnersley and Peters (18) increased the stability of the pink colors obtained with thiamine, the test was unsatisfactory for colorimetric work because of the yellow tinge which persisted. Even though Kinnersley and Peters were not successful in placing

the reaction on a colorimetric basis, their formaldehyde-azo test was a definite advancement toward developing a colorimetric test for thiamine, which minimized the coupling of histamine type compounds with diazotized sulfanilic acid.

Deviatmin (19) modified the Kinnersley-Peters test by heating the reagent with the vitamin test solution before comparing with standards. This modification may prove to be of doubtful value, since diazotized sulfanilic acid decomposes upon heating.

Since Ehrlich (20) found diazotized sulfanilic acid useful as a reagent, many workers have sought to use this compound for coupling with substances of biochemical interest. It was surmised that the investigation of other derivatives of aniline or the naphthylamines might afford a reagent of greater specificity for thiamine than diazotized sulfanilic acid. A diazotized amine which would react with the vitamin to form a colored water-insoluble compound would afford the possibility of separating this entity from extraneous material, and so provide a better basis for a chemical estimation. A reagent which would not react with other biologically important compounds but only with thiamine to produce a highly colored precipitate would be ideal. Our search for such a reagent has, we believe, been successful.

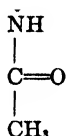
EXPERIMENTAL

A great many amines were diazotized according to the method of Kinnersley and Peters (18), which involved the use of sodium nitrite and a solution of sulfanilic acid in hydrochloric acid. We employed one molecular equivalent of the amine tested in place of sulfanilic acid.

The attempts to couple thiamine with diazotized amines in an acid solution to produce colorations or colored precipitates were unsuccessful. This confirmed the experience of Kinnersley and Peters (18). When solutions of the diazotized amines were made alkaline and added to aqueous test solutions of thiamine, coupling took place, as evidenced by a series of color changes. Similar experiments were carried out simultaneously with test solutions of histidine, since it was most desirable to find a diazotized amine which would differentiate between this compound and the vitamin. The diazotized naphthylamines and the diazotized derivatives of

aniline coupled in an alkaline solution with thiamine to produce a variety of red colors, whereas histidine coupled under similar conditions to produce yellow- and orange-colored solutions.

Of the amines investigated the alkaline diazotates of *p*-aminoacetanilide and *p*-aminoacetophenone produced purple-red-colored precipitates with solutions containing thiamine.



p-Aminoacetanilide

p-Aminoacetophenone

A brief preliminary report of these reagents has been made (21).

To bring about a diazotization reaction with *p*-aminoacetanilide, it was first necessary to dissolve this compound in hydrochloric acid as a preliminary step. Upon standing, acid solutions or dilute aqueous solutions of this amine decompose and assume a deep red color. The decomposition takes place in the dark as well as in the light. Evidently a hydrolysis takes place slowly at the point where the acetyl radical is attached to the secondary amino group in the molecule.

Since *p*-aminoacetophenone is fairly stable in aqueous solutions, our subsequent work was confined to this amine which proved to satisfy the requirements for its use as a reagent for thiamine. In our hands this reagent is best prepared from the following three solutions, freshly mixed before using.

Solution A—3.18 gm. of *p*-aminoacetophenone (Eastman Kodak Company, No. 631) are dissolved in 45 cc. of concentrated hydrochloric acid (37 per cent) and made up to a final volume of 500 cc. in a volumetric flask with distilled water. Precautions are taken to keep the solution in a glass-stoppered flask and to avoid the presence of strong light when not in use. The activity of this solution is unaltered after standing for 6 months.

Solution B—22.5 gm. of c. p. sodium nitrite are dissolved in distilled water and made up to a final volume of 500 cc. in a glass-

stoppered volumetric flask. This reagent begins to deteriorate after standing for a month. It is best preserved at refrigeration temperatures.

Solution C—20 gm. of c. p. sodium hydroxide are dissolved in 600 cc. of distilled water; 28.8 gm. of c. p. sodium bicarbonate are then added and the solution made up to a final volume of 1 liter with distilled water.

Directions for Diazotization—The diazotization reaction is carried out in an ice bath with use of 1 part by volume each of Solution A and of Solution B. This reaction requires a period of about 10 minutes. The mixture is agitated by means of an electric stirrer. At the end of this period 4 parts of Solution B are added to the resultant mixture. The solution is stirred and maintained at a temperature of 0–5° for at least 20 minutes. When the reaction is completed, the diazotized solution remains satisfactory for use at least 12 hours at refrigeration temperatures. It is advisable to prepare fresh solutions daily.¹

Directions for Making Final Reagent—In making the final reagent for reaction with the vitamin, add 20 cc. of freshly diazotized *p*-aminoacetophenone to a flask containing 275 cc. of Solution C. When these reagents are mixed, a purple coloration appears. This color disappears on stirring with an electric stirrer for a period of 5 to 10 minutes. The test solution must be adjusted to pH 5.0 to 6.0 immediately before it is allowed to react with the final reagent.

For a simple qualitative test on a solution containing thiamine, small Wassermann tubes are well suited for carrying out the reaction. 2 cc. of final reagent were added to 1 cc. of the thiamine test solution. The reaction is characterized by the formation of a purple-red coloration within 20 minutes and upon standing a precipitate of the same color settles out. For solutions containing less than 5 micrograms per cc. it is necessary to use larger quantities of the test solution. With smaller quantities of the vitamin, a small hand-glass may be necessary for observing the colored precipitate which settles out within 20 to 24 hours. By using the hanging drop technique, as little as 0.1 microgram of synthetic thiamine may be detected under the microscope.

The purple-red precipitate is flocculent and exhibits the follow-

¹ It has been found that this reagent retains its activity for a week.

ing properties: it is insoluble in water, concentrated alkali, dilute acids, and petroleum ether; is soluble in 95 per cent ethyl alcohol, propyl, butyl, and isobutyl alcohol, acetone, dioxane, glacial acetic acid, ethyl ether, carbon tetrachloride, chloroform, benzene, toluene, and xylene.

Of the solvents mentioned, isobutyl alcohol, toluene, and xylene were found best for extracting the colored suspension to form a colored non-aqueous liquid phase. Of these latter solvents, the aromatic compounds seemed to possess the best working properties for quantitative work, since they are least soluble in water.

The reagent has been tried successfully with the formation of the characteristic purple-red precipitate on samples of defatted wheat germ, defatted rice polishings, Seidell's international adsorbate, Anheuser-Busch yeast concentrate No. 2, Anheuser-Busch yeast adsorbate, Eli Lilly rice polishings adsorbate No. 928,389, crystalline thiamine (Merck), both natural and synthetic, as well as synthetic thiamine crystals supplied by the Winthrop Chemical Company. It was found that the colored precipitate could be removed from extraneous material and recovered by extraction with acetone and evaporation of this solvent with the subsequent addition of water.

In order to ascertain whether or not the new reagent reacted with other biologically important compounds, it was tested with the following substances: acetic acid, acetone, adrenalin, adenine, albumin, ascorbic acid, betaine, caffeine, cytosine, casein hydrolysate, creatine, creatinine, dextrose, ethyl alcohol, fructose, galactose, gelatin hydrolysate, glutathione, guanidine, guanine, hydroquinone, inositol, insulin, lactose, methyl alcohol, nicotinic acid, nicotinic acid amide, nucleic acid, oxalic acid, phenol, phloroglucinol, quinine, resorcinol, riboflavin, saccharin, sarcosine, sucrose, theelin, thioneine, thiochrome, trimethylamine, thymine, tyramine, uracil, urea, uric acid.

Each of the twenty-two amino acids was also tested. Of all the substances tested, inositol (ash-free, Eastman, No. 1252) was the only compound which produced a colored precipitate with the reagent. The precipitate obtained with this compound was flocculent and greenish blue in color. When this precipitate was collected on filter paper and washed with distilled water, it changed to a yellow color. It was soluble in dilute acids and slightly in

distilled water. The inositol precipitate is insoluble in dilute alkali. In aliphatic solvents it imparted a slight yellow coloration; in aromatic solvents it is insoluble. Inositol did not interfere with the reaction when thiamine was allowed to react with the reagent, since its reaction product could be removed following filtration by washing with dilute acid.

In an attempt to elucidate the mechanism of the reaction involved in the formation of the purple-red precipitate obtained by allowing thiamine to react with the reagent, some of the following intermediate compounds used in the synthesis of thiamine were studied: Merck, 37-AR-84(2), 2-methyl-5-bromomethyl-6-aminopyrimidine hydrobromide; Merck, 37-AR-85(3), 4-methyl-5- β -hydroxyethylthiazole; Merck, 37-AR-86(1), 5-ethoxymethyl-6-aminopyrimidine; Merck, 37-AR-105, 4-methyl-5- β -hydroxyethylthiazole methiodide.

Both pyrimidine derivatives produced a yellow coloration when permitted to react with the final reagent, whereas the thiazoles produced purple-red precipitates whose properties were similar to the colored precipitate obtained with vitamin solutions. It is of interest to note that the free thiazole portion of the molecule reacted much more slowly and produced a smaller amount of precipitate than an equivalent quantity of the quaternary form, which reacted almost instantaneously.

The following synthetic compounds were obtained from Dr. A. R. Todd of the Lister Institute, London, and were tested with the reagents: 4-methylthiazole, 3-(6'-amino-4'-ethylpyrimidyl-5')-4-methyl-5- β -hydroxyethylthiazolium hydrochloride, 3-(6'-amino-4'-methylpyrimidyl-5')-4-methylthiazolium chloride hydrochloride, 4-amino-5-aminoethyl-2-methylpyrimidine hydrochloride, 3-(6'-amino-2'-methylpyrimidyl-5'-5')-methyl-4-methylthiazolium chloride hydrochloride, 3-(6'-hydroxy-2'-methylpyrimidyl-5')-methyl-4-methylthiazolium chloride hydrochloride.

The only compound in this group which reacted with the reagent to produce a purple-red precipitate was the inactive isomer of thiamine, 3-(6'-amino-4'-ethylpyrimidyl-5')-4-methyl-5- β -hydroxyethylthiazolium chloride hydrochloride. It is of interest to note that this compound was similar in its reactivity to the free thiazole portion of the vitamin which reacted much more slowly with the reagent than the vitamin itself, which contains a methyl-

ene bridge between the thiazole and pyrimidine portions of the molecule.

It was also found that under controlled conditions the colored precipitate, obtained by allowing the reagent to react with thiamine, obeyed Beer's law in aliphatic as well as aromatic solvents.

The absorption spectra² of purified samples of colored precipitates prepared with the reagent from Eli Lilly adsorbate (rice polishings), as well as Merck and Winthrop synthetic thiamine, indicated a maximum absorption of 325 $m\mu$ for all samples; in the visible region the Winthrop and Merck products exhibited identical maxima at 516 $m\mu$, whereas the precipitate obtained from the Eli Lilly adsorbate exhibited the same type of absorption but with a maximum at 520 $m\mu$. This may have been due to small quantities of impurities present in the product obtained from the adsorbate; nevertheless the results check within the limits of photographic technique and indicate that the reaction products obtained from natural sources are similar to those obtained by reaction of the reagent with the synthetic vitamin.

DISCUSSION

The reagent described herein seems to be specific for the 4-methyl-5- β -hydroxythiazole portion of the thiamine molecule. In applying this reagent for quantitative work, we have had considerable difficulty in obtaining approximately quantitative extraction of the vitamin from such biological materials as yeast, wheat germ, etc. After extraction, the problem of concentrating the vitamin without destruction presented itself. Adsorbates seem best suited for this purpose, since they also allow the removal of extraneous material without destruction of the vitamin. It should be mentioned that adsorbates had to be specially treated so that eluates made therefrom did not carry along inorganic substances which interfered with the activity of the reagent. High salt concentrations and metallic ions were found to influence the effectiveness of the reagent. In working with adsorbates from extracts of natural products it is difficult to obtain 100 per cent elution of the vitamin.

These obstacles must be overcome before a method generally

² The authors are indebted to Dr. C. E. Bills and Dr. F. G. McDonald of Mead Johnson and Company for the absorption spectra studies.

applicable can be worked out for using the new reagent as part of a quantitative chemical procedure for the assay of foodstuffs and biological materials. Further work along these lines is in progress in this and other laboratories.

SUMMARY

Two reagents have been described which react with the thiazole portion of thiamine to form purple-red compounds which are highly insoluble in water but soluble in selective organic solvents. Alkaline diazotized *p*-aminoacetophenone was found to be the better of the two reagents reported. Many obstacles, such as complete quantitative extraction and concentration of thiamine from biological materials, must be overcome before the reagent is applicable in quantitative work.

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CHEMICAL DETERMINATION OF VITAMIN B₁

I. REACTION BETWEEN THIAMINE IN PURE AQUEOUS SOLUTION AND DIAZOTIZED *p*-AMINOACETOPHENONE

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In the recent literature several chemical methods for the determination of thiamine are described. Some of these admittedly lack specificity (1-3). The determination of the blue fluorescence in ultraviolet light of thiochrome, one of the oxidation products of thiamine, has been most widely investigated (4-12). Its application to the quantitative determination of thiamine is complicated by similar blue fluorescence given by other substances (4-7, 9), the difficulty in correcting for such non-specific fluorescence (6, 13), the presence in biological materials of substances which may combine with thiochrome to form non-fluorescent compounds (7) or which may affect the intensity of the fluorescence (14, 15), the instability of the fluorescence during measurement (16), and the fact that thiochrome is an intermediate in the oxidation of thiamine (4-8), the yield of which must be influenced by other oxidizing and reducing substances (4, 6-8) as well as the amount of thiamine and reagent present (4-7).

In 1934, Kinnorsley and Peters (17) reported that the reaction between thiamine and diazotized sulfanilic acid in alkaline solution was made more sensitive by the presence of formaldehyde. This reaction has been repeatedly criticized because of lack of specificity. Their procedures were subsequently improved (18) so that semiquantitative data were obtained when the reaction was applied to the determination of thiamine in biological materials. Prebluda and McCollum (19) modified the reagent using diazotized *p*-aminoacetophenone; the formaldehyde addition was

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not included in the test. The diazonium salt of 2,4-dichloroaniline has also been reported (20) as a reagent for the chemical determination of thiamine in biological materials. The specificity of this reagent for the vitamin and the adequacy of the procedures used in its application require confirmation.

In the present studies we have investigated the use of diazotized *p*-aminoacetophenone as a reagent for the chemical determination of thiamine. The method has been found specific for the vitamin, made quantitative, more sensitive, and successfully applied to analyses of a number of natural sources of thiamine, including yeast, rice polish, wheat germ, and liver.

EXPERIMENTAL

Thiamine reacts with diazotized *p*-aminoacetophenone in alkaline solution to produce an insoluble red pigment. In our investigations we have used this reagent exactly as described by Prebluda and McCollum¹ (19). For the estimation of small amounts of the vitamin, a solvent was sought which would extract quantitatively the thiamine derivative to yield a solution suitable for colorimetric evaluation. The solvent should not only extract quantitatively the pigment from the aqueous phase, but should also be completely immiscible with water, differ appreciably in specific gravity so that the separation into the two phases may take place with satisfactory rapidity, possess a low vapor pressure in order that no change in volume of the solution may occur during the colorimetric evaluation, and finally be a stable, non-reactive compound so that the character of the reaction product may not be altered by its use. Xylene possesses all of these qualifications and has been used throughout for this purpose.

Specificity of Method for Detecting Thiamine—Inasmuch as we always use the technique of adsorption on synthetic zeolite (permutit) and elution in preparing concentrates for testing, only those compounds similarly adsorbed were studied. A series of tests was conducted with 5 mg. quantities of a number of such

¹ We are very much indebted to Dr. E. V. McCollum for supplying us with detailed directions for the preparation of the reagent, prior to publication of the complete report.

compounds² (21). The compounds investigated were ammonium nitrate, hydroxylamine, hydrazine, ethylamine, trimethylamine, benzylamine, choline, guanidine, atropine, pilocarpine, nicotine, quinine, histamine, adrenalin, tyrosine, histidine, arginine, and lysine. Of these compounds some did react with the reagent to yield colored derivatives. Histamine and histidine both gave orange colors; tyrosine, pink; and adrenalin, red, which changed to violet and finally faded to yellow. However, none of these colored derivatives was extracted by the xylene but all remained in the aqueous phase.

Degradation products of thiamine which are not biologically active were found to produce no color with the diazonium salt. This was true after sulfite cleavage which yields intact pyrimidine and thiazole derivatives (22), after the action of alkali and heat which results in the opening of the thiazole ring (23), after simple deamination, and after oxidation to the thiochrome stage (13). Because in both of these last two reactions the free amino group on the pyrimidine ring no longer exists, Barger and associates (13) believe that it is this group which couples with diazonium salts to yield colored derivatives.

Probably the best proof of specificity of the method is derived from tests conducted on a number of biological materials (24). In all cases the substance responsible for the red color in the xylene layer was found to be as completely adsorbed and eluted as thiamine and also as unstable to alkali and heat.

Time Required for Completion of Reaction—100 micrograms of thiamine chloride in 10 cc. of water at pH 7 were allowed to react at room temperature with 20 cc. of the Prebluda-McCollum reagent for variable periods of time. The reaction product was then extracted³ in each case with 2 cc. of xylene and the color evaluated in a microcolorimeter with the 24 hour sample as the standard for comparison. The results of this study are presented in Fig. 1. The reaction was observed to have gone 75 per cent to completion within 5 to 30 minutes and by the 13th hour maximal and constant values were obtained. In all our subsequent

² Dr. Howard B. Lewis of the Department of Biological Chemistry, University of Michigan, kindly supplied us with many of these compounds.

³ Cork stoppers are used.

tests the xylene extraction was carried out after the reaction had been allowed to proceed for at least 15 hours (overnight).

Influence of Variations in Volume of Thiamine Solutions upon Reaction—A series of tests was performed in which 20 cc. of the reagent were added to vitamin solutions containing in all cases 100 micrograms of thiamine chloride but varying in volume from 1 to 20 cc. The results of these tests are given in Fig. 2. When comparison is made with the standard vitamin solution, 10 cc. in volume, an increase in the recovery of the vitamin is noted in the more dilute solutions and *vice versa*.

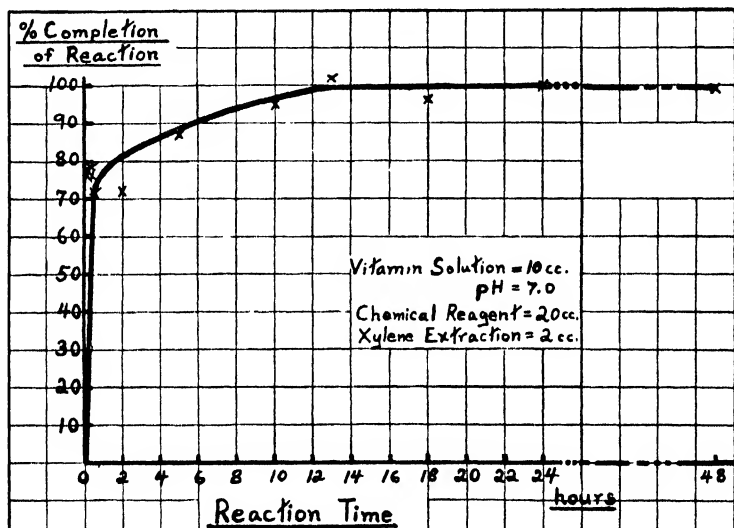


FIG. 1. Time required for completion of the reaction between thiamine and diazotized *p*-aminoacetophenone. A total of 100 micrograms of thiamine chloride was used in each test.

In similar fashion, tests conducted with varying quantities of the reagent but with all other factors maintained constant indicate that greater recoveries of the vitamin derivative are possible when decreasing amounts of the reagent are used. This is true to within certain limits, after which the quantity of the reagent may be insufficient to couple with all of the vitamin. These variable recoveries were found to be due to the fact that the thiamine concentrations are determined by an alkaline reagent which

tends to destroy the vitamin and as the ratio of the volume of the reagent to that of the vitamin solution is decreased this destruction is reduced. These findings are presented in Fig. 3.

In order to minimize the destructive action of the alkaline reagent upon the vitamin, tests were conducted at 0–5° with all solutions initially chilled to this temperature range. Only 70 per cent recoveries were obtained after a 24 hour reaction period, probably owing to a decrease in the velocity of the chemical reaction. Attempts to make a reagent containing the diazonium

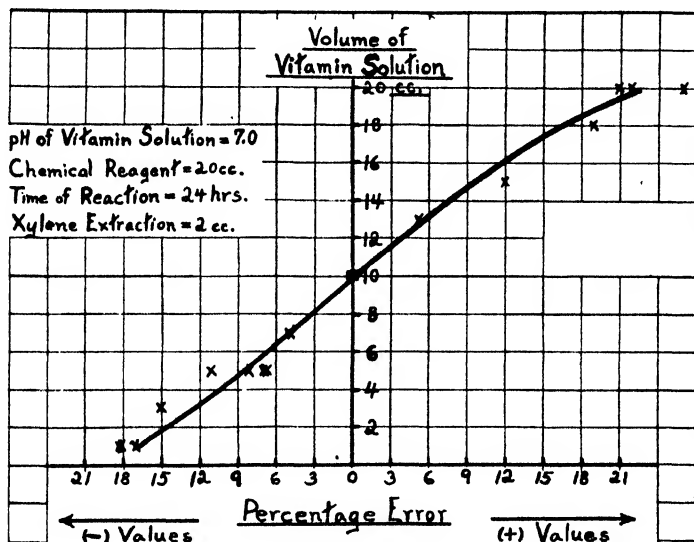


FIG. 2. Influence of variations in volume of the thiamine solution upon the reaction between thiamine and diazotized *p*-aminoacetophenone. A total of 100 micrograms of thiamine chloride was used in each test.

salt of *p*-aminoacetophenone in a less alkaline solution were unsatisfactory.

We have preferred to use the proportion of 2 parts of the reagent to 1 part of the vitamin solution because tests on biological materials have indicated that other substances are always present in the final test solutions, which also react with the reagent, so that an excess of reagent is desirable.

Influence of pH upon Reaction—In the reaction between diazotized *p*-aminoacetophenone in alkaline solution and thiamine the

initial hydrogen ion concentration of the latter solution was found to exert a profound effect upon the percentage recovery of the vitamin derivative. In the investigation of this problem 142 tests were conducted. The most significant series of these determinations is presented in Fig. 4. Pure aqueous solutions of the vitamin, containing in all cases 100 micrograms of thiamine chloride but varying amounts of acid or alkali, were tested. All other factors influencing the reaction were maintained constant.

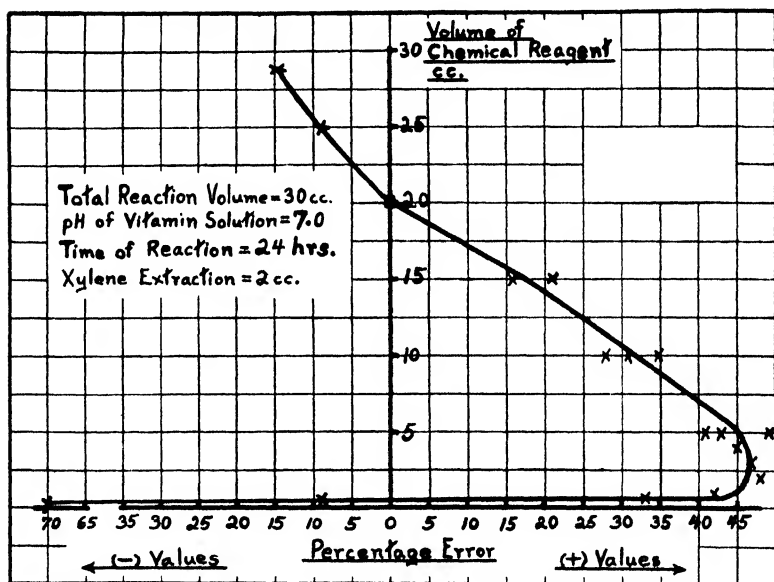


FIG. 3. Effect of varying quantities of the reagent upon the reaction between thiamine and diazotized *p*-aminoacetophenone. A total of 100 micrograms of thiamine chloride was used in each test.

Examination of Fig. 4 indicates that, as the acidity of the vitamin solution is decreased, greater recoveries of the vitamin derivative are obtained. For maximal and constant recoveries it is necessary to neutralize to a point just alkaline to litmus and to follow this *immediately* with the addition of the reagent. If the solution is made too alkaline, losses as large as 10 per cent may occur despite the immediate addition of the reagent. The variable results obtained on the alkaline side are probably due not to any

effect of pH upon the reaction but to a destruction of the thiamine; such an interpretation is indicated in Fig. 4 by the broken line.

Reproducibility of Method—In all cases the volume of the thiamine solutions was 10 cc., the reaction was neutral to litmus, 20 cc. of the reagent were added, and after a 24 hour reaction period at room temperature the vitamin derivative was extracted by xylene. The results of this study are summarized in Table I.

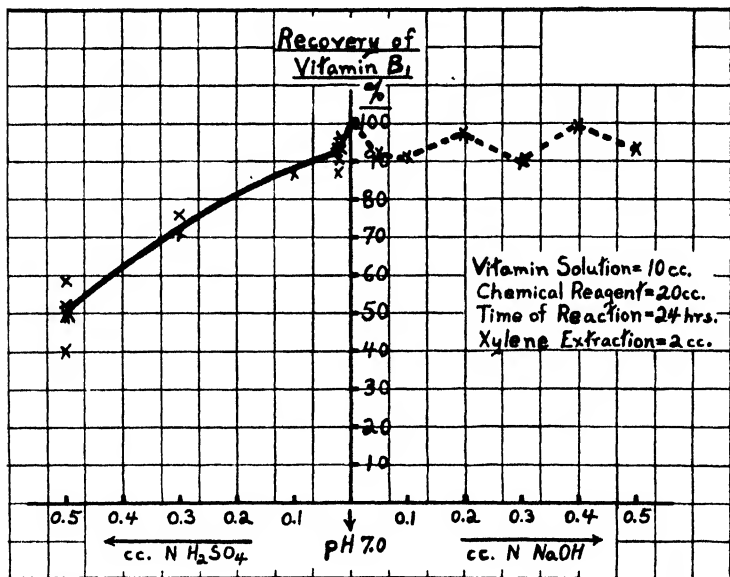


FIG. 4. Influence of pH upon the reaction between thiamine and diazotized *p*-aminoacetophenone. A total of 100 micrograms of thiamine chloride was used in each test. The variable results on the alkaline side (broken line) are probably not due to any effect of pH upon the reaction, but to a destruction of the thiamine.

In this series the maximal deviation from the average is ± 4 per cent, with the average deviation ± 2 per cent. The greatest differences are found in the tests conducted with the solutions containing 5 micrograms of thiamine chloride per cc. This concentration of the vitamin represents the lower limits of the sensitivity of the method for accurate analyses without the modifications to be described (24).

Intensity of Color Obtained As an Index of Thiamine Concen-

tration—With the solutions described in Table I as standards for comparison, tests were conducted under the standardized conditions given above to determine the vitamin concentrations in solutions ranging from 0.4 to 2.0 times the standard. Table II presents the results of the study. The curves, obtained with the

TABLE I
Reproducibility of Chemical Method for Determination of Thiamine

Thiamine concentration micrograms per cc.	No. of determinations	Deviation from average	
		Maximal per cent	Average per cent
5	12	±6	±3.5
10	12	±3	±1.5
20	12	±3	±2
40	12	±4	±1.5
80	12	±3	±2
Average.....		±4	±2

TABLE II
Chemical Determination of Thiamine Concentration in Solutions Ranging from 0.4 to 2.0 Times the Standard

Thiamine concentration in standard micrograms per cc.	Ratio of thiamine concentration in sample to standard solution								
	0.40	0.60	0.80	1.00	1.20	1.40	1.60	1.80	2.00
Colorimetric readings*									
mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
10	59.0	33.6	25.1	19.8	16.7	14.1	12.2	11.0	9.9
20	49.0	34.2	24.9	19.9	17.0	13.9	11.7	10.7	9.6
40	54.0	36.8	25.9	19.7	16.7	13.4	11.7	9.7	8.8
80	65.2	38.4	26.4	20.4	15.9	12.6	10.7	8.6	7.5
Theoretical	50.0	33.3	25.0	20.0	16.7	14.3	12.5	11.1	10.0

* Standard solutions of the vitamin derivative in xylene set at 20 mm.

10 and 20 micrograms per cc. solutions as the standards, show good agreement with the theoretical values. With the 40 and 80 micrograms per cc. solutions as standards, discrepancies occur which become greater as the differences between sample and standard increase. However, in all of these tests such disagree-

ment does not indicate errors in the observed values, because of the excellent reproducibility of the individual determinations. It indicates that, for estimating the thiamine concentration by this method without the modifications to be described (24), it is essential to have available a previously determined reference curve bracketing the value obtained.

SUMMARY

The characteristics of the reaction between thiamine in pure aqueous solution and diazotized *p*-aminoacetophenone with respect to specificity, time for completion of the reaction, influence of variations in volume and pH of the solutions, reproducibility, and applicability to the quantitative determination of the vitamin concentration were studied. Xylene was found to be a selective solvent for the quantitative extraction of the reaction product. With the use of xylene, the reaction is specific for thiamine and with controlled conditions may be used for the quantitative determination of it in pure aqueous solution.

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CHEMICAL DETERMINATION OF VITAMIN B₁

II. METHOD FOR ESTIMATION OF THE THIAMINE CONTENT OF BIOLOGICAL MATERIALS WITH THE DIAZOTIZED *p*-AMINOACETOPHENONE REAGENT

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In Paper I (1) the reaction between thiamine in pure aqueous solution and diazotized *p*-aminoacetophenone (2) was investigated and found to be suitable as the basis of a chemical method for the determination of the vitamin. For the application of the reaction to analyses of biological materials, it is first necessary to concentrate the thiamine into a small enough volume so as to be within the limits of sensitivity of the method. This in itself presents little difficulty but the problem was found to be complicated by the fact that various impurities in solutions of thiamine completely inhibit the reaction. It was necessary either to modify the reaction so that it would not be subject to such inhibition or to devise methods for preparing concentrates which would eliminate the interfering substances from the solution to be tested. Both of these requirements have been satisfied to such an extent that accurate determinations of thiamine in biological materials are possible, with the diazotized *p*-aminoacetophenone reagent.

EXPERIMENTAL

Advantages of Conducting the Reaction in Presence of Phenol and Ethyl Alcohol—In studies involving the use of phenol for extracting the vitamin from saturated salt solutions recoveries greater than 100 per cent were consistently obtained owing to the presence of traces of ethyl alcohol or of phenol, which escaped

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ether extraction, in the final concentrates. Other organic hydroxy compounds such as methanol, benzyl alcohol, and pH indicators also increase the sensitivity of the reaction.

In order to investigate the behavior of phenol in this respect, a series of determinations was made in which varying amounts of phenol were added to the vitamin solutions, each 10 cc. in volume and containing a total of 100 micrograms of thiamine chloride. No phenol was added to the standard solution. The procedure used in testing for the vitamin was the same as that described in our experiments conducted with pure aqueous solutions of thiamine (1). Although phenol, like some other organic compounds, reacts with the reagent to yield a colored solution, only the red pigment due to the vitamin derivative is extracted by the xylene. The results of these determinations are presented in Fig. 1, A.

It is apparent from the curve in Fig. 1, A that small quantities of phenol increase greatly the sensitivity of the reaction but with larger amounts of phenol the intensity of the color in the xylene layer becomes relatively constant. In the presence of very large amounts of phenol (300 mg. or more) the reaction fails unless larger quantities of the reagent are used.

Ethyl alcohol used in place of phenol affects the reaction similarly. When both phenol and alcohol were used, there was an increase in the intensity of the color in the xylene layer appreciably greater than that observed when the reaction was conducted in the presence of either of these substances alone. Fig. 1, B illustrates these findings.

With appropriate quantities of phenol (50 mg.) and ethyl alcohol (equal in volume to that of the thiamine solution), results in close agreement with the theoretical values were obtained in tests conducted with solutions ranging from 0.4 to 5 times the standard, in this case also the 10 micrograms per cc. solution of thiamine chloride. For amounts more than 2 times the standard, greater quantities of xylene were used in order to obtain solutions the color intensities of which were comparable to the standard. Without the phenol and alcohol it had previously (1) been necessary to employ a standard more closely approximating the test solution in concentration, and in some cases a previously determined reference curve had to be used in order to obtain the true value.

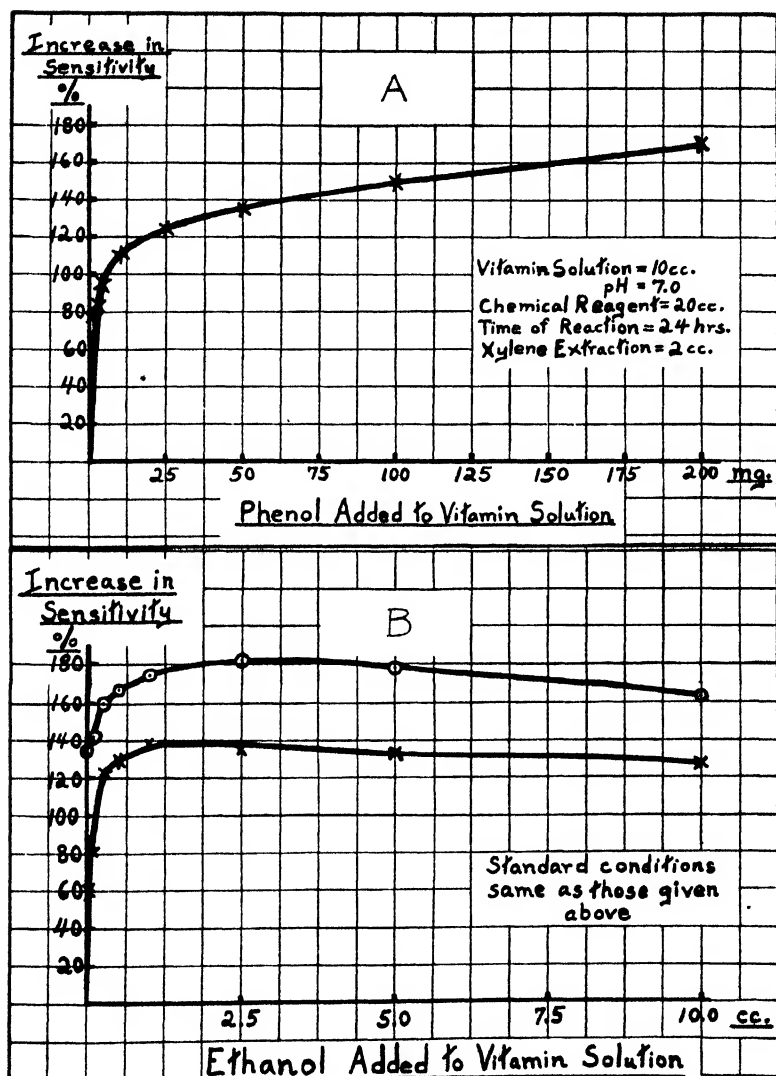


FIG. 1. Use of phenol (A) and ethanol (B) to increase the sensitivity of the reaction for the chemical determination of thiamine. A total of 100 micrograms of thiamine chloride was used in each test. In B the crosses represent values obtained with varying amounts of ethanol alone; the circles, values obtained with 50 mg. of phenol present in each case in addition to the ethanol.

Because of the increased pigment production in the presence of phenol and alcohol, it became possible, by using three-tenths quantities of test solution and reagent but the same volume of xylene, to determine accurately smaller quantities of the vitamin, as little as a total of 10 micrograms of thiamine chloride. Quantities as small as 5 micrograms may be estimated with the average error not exceeding ± 10 per cent.

It was also found that the presence of phenol and alcohol made possible quantitative analyses of solutions containing substances which otherwise would inhibit the reaction. 100 per cent recoveries of the vitamin from concentrated salt solutions became possible, permitting the use of such solutions for elution from permutit without subsequently separating the vitamin from the salts as had previously been necessary. The advantage of the use of both phenol and alcohol and a larger volume of alcohol than that which had been previously observed to be optimal (one-fourth of the vitamin solution) in tests on pure aqueous solutions is indicated in Table I.

The presence of phenol and alcohol has the further advantage that effects of traces of substances in the final solution which would have a similar action are no longer apparent. Thus, it makes possible the use of an inside indicator for the adjustment of the pH of the thiamine solution before the addition of the reagent. (Thymol blue has proved satisfactory for this purpose, the color due to the indicator not being extracted by xylene.) It has also been found to minimize errors (1) due to deviation from standard conditions, such as variations in volume of the vitamin solution or reagent, and to exert an appreciable protective effect on the vitamin in solutions alkaline to litmus.

Use of Adsorption and Elution Technique for Preparation of Thiamine Concentrates—For the preparation of suitable test solutions from biological materials various methods of extraction, adsorption, and elution were tried. Lloyd's reagent was not satisfactory, since eluates (3, 4) from it contained unknown interfering substances. Repeated extractions of the Lloyd's reagent with the eluting solution (4) did not remove entirely these interfering substances.

Synthetic zeolite (permutit), first used by Cerecedo and Hennessey as an adsorbent for vitamin B₁ (5), was found to be more

suitable. These authors later employed a hot 25 per cent KCl solution in order to elute the vitamin from the zeolite (6). Rather than a simple KCl solution, we have used one acidified with H_2SO_4 to pH 2 for the elution procedure. When the salt solution at pH 7 was employed, recoveries of from 90 to 95 per cent of the theoretical values were obtained. This, we believe, is due to the lability of the vitamin to heat even at pH 7. The successful

TABLE I

Use of Phenol and Ethanol to Allow Quantitative Determination of Thiamine Content of Concentrated Salt Solutions

Salt present	Concentration of salt*	Concentration of phenol*	Ethanol added*	Percentage recovery of thiamine†
	gm. per cent.	mg. per cent		
Na_2SO_4	20	0	None	40
NaCl	20	0	"	42
KCl	25	0	"	65
Na_2SO_4	20	5	"	77
NaCl	20	5	"	83
KCl	25	5	"	85
Na_2SO_4	20	0	Equal volume	91
NaCl	20	0	" "	94
KCl	25	0	" "	88
"	25	0	0.25 volume	48
"	25	0	0.25 "	45
Na_2SO_4	20	5	Equal volume	100
NaCl	20	5	" "	102
KCl	25	5	" "	98

* These substances were added to the thiamine solution before the reagent.

† Each of the solutions contained 100 micrograms of thiamine chloride.

elution of thiamine by 3.5 N H_2SO_4 (7) indicates that zeolite holds the vitamin by adsorption rather than by a base exchange as suggested by others (5, 6, 8). Obviously, a strong acid solution cannot enter into a base-trading reaction. We have therefore continued to refer to the procedure with zeolite for the preparation of thiamine concentrates as adsorption and subsequent elution. Supporting evidence for this view is found in Paper III

(9) dealing with the behavior of aqueous solutions of the phosphoric esters of thiamine when subjected to the same adsorption and elution procedures.

The limiting factor in the method for the chemical determination of thiamine appears to be the efficiency of the zeolite adsorption. The presence of salts, non-aqueous solvents, and large concentrations of other organic material prevents quantitative adsorption of thiamine. Only 65 per cent recoveries of the vitamin were obtained by adsorption and elution when the thiamine solutions (100 cc.) contained NaCl in amounts comparable to an equal volume of urine and buffered to yield a similar titratable acidity. In the presence of large amounts of ethyl alcohol, acetone, or phenol the recoveries were exceedingly small. Hence, it is necessary that the vitamin extract be as free as possible from substances which interfere with the adsorption.

Methods for Quantitative Extraction of the Vitamin from Biological Materials—Since thiamine exists in biological materials both in the free state and as the phosphoric esters (10, 11), a method by which both forms can be extracted is desirable. A simple aqueous extraction is preferable if the resulting solution allows quantitative adsorption of the vitamin. Aqueous and 80 per cent methyl alcohol solutions are capable of extracting both forms of the vitamin from biological materials.

The 80 per cent methanol solution is a more selective solvent, since it eliminates considerable amounts of salts and organic material from the final solution. However, because zeolite will not adequately adsorb thiamine from such a solution, the alcohol must be removed by *in vacuo* distillation. In some cases a considerable amount of extracted lipids may come out of solution during the alcohol distillation, necessitating an additional ether extraction in order to yield a suitable concentrate.

Of the water-immiscible solvents investigated by Greene and Black, benzyl alcohol and phenol proved to be the most satisfactory (12). They report that the distribution coefficient of the vitamin between solvent and water is markedly increased by the saturation of the aqueous phase with NaCl. Inasmuch as ethyl alcohol precipitates cocarboxylase from aqueous solution,¹

¹ Personal communication from Dr. Joseph L. Melnick and Dr. Kurt G. Stern of the Laboratory of Physiological Chemistry, Yale University, New Haven.

we believe that these solvents also are unable to extract phosphorylated thiamine from biological materials. To use such solvents, a preliminary conversion of cocarboxylase to thiamine is probably essential.

Phenol (88 per cent solution) has an extremely high solvent action for the vitamin. However, recovery of the thiamine from the phenol solution, where it is apparently combined as a phenolate, requires a laborious procedure (13).

Benzyl alcohol is a more selective solvent for thiamine than phenol. Salts are practically insoluble in it and many organic compounds soluble in phenol are not extracted. The vitamin may be recovered from the alcohol phase by adding acidulated (pH 3 to 4) water and an excess of ether (5 volumes). *In vacuo* distillation will remove all dissolved ether from the separated aqueous phase.

Chemical Determination of Thiamine in Biological Materials—In the present study analyses of rice polish, wheat germ, yeast, and liver preparations were carried out. With the first three substances, low in salt relative to the vitamin content, simple aqueous extraction yields solutions suitable for adsorption on permutit. A sample sufficient to yield an extract containing approximately 150 micrograms of thiamine is taken for analysis. 20 to 30 parts of water at 70° are added to the material previously ground to a powder, the pH is adjusted to 4.5, and the extraction allowed to proceed for 30 minutes under an atmosphere of nitrogen with the water bath at 70–72°² and with mechanical stirring. The suspension is centrifuged and the pooled extract and washings subjected to zeolite adsorption. 80 per cent methyl alcohol is equally efficient for the extraction. The same procedure may be followed when this solvent is used. An extraction period of 10 minutes is satisfactory.

These extraction methods cannot be employed in the analyses of all types of biological materials because the resulting solutions may contain other substances which prevent quantitative adsorption of the vitamin by the zeolite. In analyses of a liver

² Dr. Harold Levine of the Premier-Pabst Corporation, Milwaukee, has used a similar procedure for the quantitative extraction of thiamine from yeast powder. We are indebted to him for a number of helpful suggestions and also for supplying us with several yeast preparations used in the course of this investigation.

extract powder³ theoretical recoveries of added thiamine were obtained only by use of benzyl alcohol. The material is dissolved in a minimal quantity of water, Na₂SO₄ added to saturation, and the solution concentrated *in vacuo* to a syrup. Benzyl alcohol is then added and the concentration continued until no aqueous phase remains. The vitamin is recovered from the pooled alcoholic extract and washings by the procedure described in the preceding section. The benzyl alcohol method for the extraction of thiamine has also been applied to analyses of urine with good results, although the procedure requires further standardization.

The thiamine solution, free from organic solvents, is subjected to zeolite adsorption and subsequent elution.

The method, by which consistently excellent results are obtained, is described below. The essential features of the apparatus used are shown in Fig. 2. A large batch (3 to 5 pounds) of the zeolite⁴ is first treated according to the directions of Cerecedo and Kaszuba (14) in order to remove free alkali. The material is then washed by suspending it successively five times in distilled water, three times in 95 per cent ethyl alcohol, once in acetone, and finally twice in anhydrous ether. The material is filtered, dried at 50°, and stored in bottles for future use. Exactly 3 gm. of the purified zeolite are poured into the filter tube (8 mm. inside diameter) on top of the glass wool pad. This gives a filtering column about 4 inches in height. Inasmuch as the same permutit filter is used repeatedly after KCl elution, except in the first instance, the zeolite is first prepared by running through it 30 cc. of a 25 per cent solution of KCl, adjusted to pH 2 and heated by the steam jacket. With the suction continued, the above is followed by a washing with 500 cc. of hot distilled water. The flow of steam is stopped and the filtering chamber opened to the nitrogen reservoir (a large rubber balloon) in order to fill the system with the inert gas. The vitamin solution at pH 4.5 (preferably non-buffered) is added to the graduate and passed by way of the siphon into the filtering column. With the suction now turned off and the apparatus open to the atmosphere at both top (A) and bottom (B) the gravity filtration is allowed to proceed at

³ Kindly furnished by Eli Lilly and Company, Indianapolis.

⁴ Decalco, approximately 50 mesh, kindly furnished by the Permutit Company, New York.

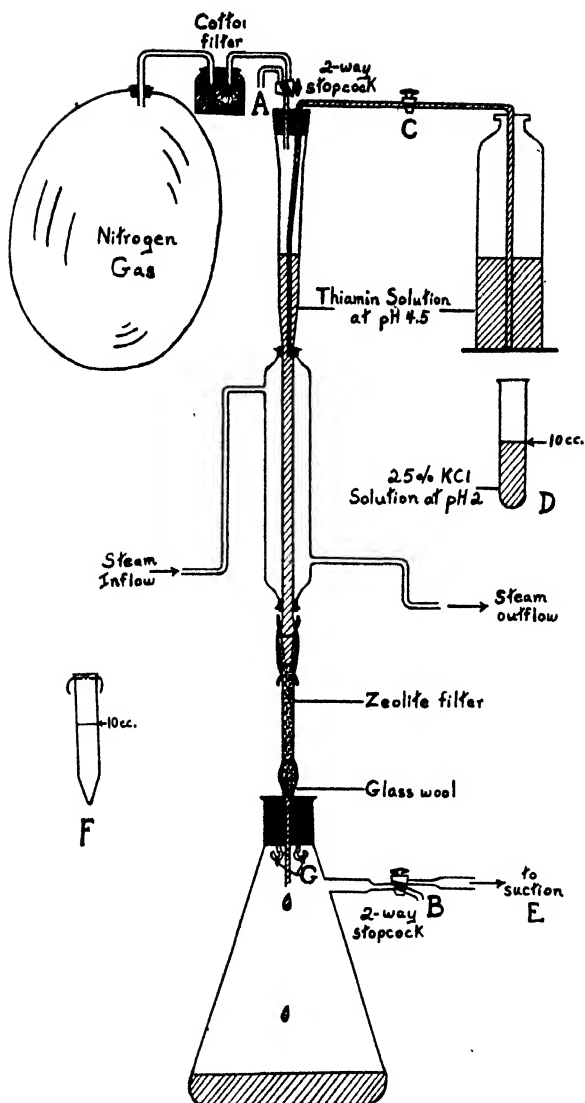


FIG. 2. Apparatus used for both quantitative adsorption of thiamine by zeolite and subsequent quantitative elution. The letters are explained in the text.

room temperature. The rate of filtration is approximately 10 cc. per minute. As soon as all of the vitamin solution is drawn into the siphon, 30 cc. of water at pH 4.5 are added to the graduate. This is then permitted to run into the siphon separated from the vitamin solution by a bubble of air until all of the vitamin solution is in the filter column. With the wash solution in position for use, the system is opened to the nitrogen reservoir and suction applied to hasten the filtration of the remaining vitamin solution through the zeolite column. The system is then opened to the atmosphere as before and with steam passing through the glass jacket, the wash solution is allowed to flow into the filtering column. The graduate is then replaced by test-tube *D* containing 10.0 cc. of the acidulated KCl solution. By using the same procedure described above this solution is siphoned into position for the subsequent elution of the vitamin. The wash solution, now heated, is then rapidly and completely drawn through the zeolite with suction applied at one end (*E*) and with the other open to the nitrogen reservoir. This solution serves the dual purposes of washing out any of the remaining vitamin solution and also raising the temperature of the zeolite filter to one optimal for elution. With the system again opened to the atmosphere and with the graduated tube (*F*) held in position (*G*) by the glass supports, the salt solution is siphoned very slowly through the steam jacket until all of it has been added. At first pressure from the nitrogen reservoir alone is used, but suction is applied at the very end to draw out all of the salt solution. The temperature of the permutit filter during the elution is about 65°. The eluate is caught in the tube (*F*) and brought to the 10 cc. mark. The permutit filter is washed with 0.5 liter of hot, distilled water and is then ready for another analysis. The entire procedure takes about 20 minutes.

3 cc. of the eluate are pipetted into a 50 cc. centrifuge bottle. This is followed by the addition of an equal volume of a 95 per cent ethyl alcohol solution containing 5 mg. of phenol per cc. 1 drop of the thymol blue indicator is added. While a fine stream of nitrogen gas is bubbled through the solution, a 1 N NaOH solution is added dropwise until a faint but positive blue color appears. 6 cc. of the Prebluda-McCollum reagent (2) are then

added. The bottle is stoppered⁵ and allowed to stand overnight at room temperature. 2 cc. of xylene are then added and the mixture shaken vigorously for 1½ minutes. After centrifugation the color in the xylene layer is compared in a microcolorimeter with a standard similarly treated. Only the color of the vitamin derivative is extracted, the aqueous phase retaining the colors due to the pH indicator, the phenolic derivative, and any other substance present reacting with the reagent to yield colored products. For the standard solution 0.5 cc. of a solution containing 50 micrograms of thiamine chloride is added to a 2.5 cc. aliquot of a blank KCl eluate (10 cc.). The blank is essential, not owing to any lack of specificity, but because the amount of pigment recovered from permutit eluates may vary from 85 to 95 per cent of the theoretical. The percentage recoveries are consistent for eluates from the same permutit filter.

A series of tests with both the permutit adsorption and elution technique and the phenol-ethanol modification of the chemical method for the determination of thiamine has indicated that consistently excellent results are yielded by the procedure. One individual may carry out as many as ten complete determinations in an 8 hour day with the chemical tests on the eluates run in duplicate. It is advisable to save all the eluates and then set up the tests as one series with one standard for comparison. A typical series of determinations with these procedures in tests conducted on pure aqueous solutions of thiamine chloride is presented in Table II. All of the tests were conducted with the same permutit filter and with one standard for comparison containing the vitamin dissolved in a blank zeolite eluate, as described. In the case of the more concentrated thiamine solution (200 micrograms per 3 cc. aliquot) only 50 micrograms were added to the aliquot, because the total amount present, 250 micrograms, represents the maximal content of thiamine chloride that can be determined by 6 cc. of the reagent under these conditions of experimentation. Thus, by the use of 6 cc. of reagent with 3 cc. of a zeolite eluate, containing a total of from 10 to 250 micrograms of thiamine chloride, quantitative results are yielded when the reaction is carried out in the presence of phenol

⁵ Cork stoppers are used.

and ethyl alcohol. When more than 250 micrograms of the vitamin are present, it is necessary to add more reagent or, as we prefer to do, dilute the solution in order to reduce the thiamine concentration below the maximal limit of the test.

When tests are conducted on the KCl eluates from zeolite, an appreciable precipitate of previously dissolved permutit appears. This precipitate does not affect the accuracy of the method when pure aqueous solutions of thiamine chloride are used. However, in tests conducted with extracts of biological materials a considerable amount of red pigment (the vitamin derivative) adheres

TABLE II

Use of Adsorption on Zeolite and Subsequent Elution As a Means for Preparing Thiamine Concentrates: Recovery Experiments

Total thiamine in 100 cc.	KCl eluate	Aliquot tested	Theoretical values		Total thiamine found	Percentage recovery
			Thiamine in aliquot	Thiamine added to aliquot		
micrograms	cc.	cc.	micrograms	micrograms	micrograms	
34	10	3	10	0	10	100
				10	20	100
84	10	3	25	0	25	100
				25	49	96
167	10	3	50	0	49	98
				50	98	98
334	10	3	100	0	106	106
				100	200	94
667	10	3	200	0	194	97
				50	246	104

to the permutit and resists xylene extraction despite the alkaline pH of the solution. A strong acid solution dissolves the precipitate, allowing the xylene to extract all of the vitamin derivative. In addition other colored compounds resulting from the reaction between substances in solution (including the phenol) and the diazonium salt are now also extracted by xylene from the acidified aqueous phase. However, on addition of an equivalent amount of alkali these other colored substances return to the aqueous phase and the permutit precipitate forms again, but the xylene this time retains quantitatively all of the color due to the vitamin derivative. After the KCl eluate has been allowed to

react with the reagent in the presence of phenol and ethyl alcohol, the major portion of the vitamin derivative is extracted by the xylene. 5 cc. of 20 per cent H_2SO_4 solution⁶ are then added and the bottles stoppered tightly (since CO_2 is liberated) and shaken for 15 to 20 seconds. After the addition of 10 cc. of 15 per cent NaOH solution, the shaking is repeated for another period of 15 to 20 seconds. The color in the xylene layer is then compared with a standard similarly treated. This modification does not lengthen appreciably the time required for the determinations. This procedure has no destructive effect on the vitamin derivative. Experiments with thiamine chloride, dissolved in blank KCl eluates of zeolite, gave the same results before and after the acidification-alkalization procedure. For the calculation of the vitamin concentration of the solution tested, no previously determined reference curve need be used, since in all cases the intensity of the color in the xylene layer varies directly as the original thiamine concentration.

Another difficulty, encountered in applying the reaction to assays of biological material, was the presence of other substances in the KCl eluates which at times exerted an appreciable inhibitory effect upon the chemical reaction. Thus, recoveries as low as 80 per cent were frequently found, whereas with pure aqueous solutions theoretical values were consistently obtained. However, the proper correction factor may be obtained and applied to give the true value for the thiamine present by determining the percentage recovery of an increment of thiamine chloride (50 micrograms) added to an aliquot of the zeolite eluate tested.

The procedures described have been used to determine the thiamine content of biological materials. Table III presents the results of these tests. The series of values obtained in the analyses of the aqueous concentrate of rich polish (Sample I) gives some index of the reproducibility of the results. Both the aqueous extraction method and method of extraction with 80 per cent methanol were used in a number of cases to prepare solutions suitable for the subsequent adsorption procedure. The close agreement between the values obtained for the initial thiamine con-

⁶ 20 cc. of concentrated H_2SO_4 (sp. gr. 1.84) diluted with distilled water to 100 cc.

centration in each case suggests that the vitamin can be quantitatively extracted by both procedures from the materials tested. In

TABLE III
Chemical Determination of Thiamine in Biological Materials

Sample	Description of preparation	Method of extraction	Chemical assay	
			Thiamine initially present*	Percentage recovery of added thiamine*
			micrograms per gm.	
A	Dried yeast powder	Aqueous	40	99
		80% CH ₃ OH	38	103
B	" " "	Aqueous	37	100
		80% CH ₃ OH	38	98
C	" " "	Aqueous	45	96
		80% CH ₃ OH	44	103
D	Artificially sweetened, concentrated aqueous extract of yeast	Aqueous	29.5	104
		80% CH ₃ OH	30.5	105
E	Dehydrated yeast and liver concentrate	Aqueous	64	99
		80% CH ₃ OH	63	105
F	50% C ₂ H ₅ OH extract of yeast powder		1.6	102
G	Aqueous extract of yeast powder with CHCl ₃ as preservative		3.9	106
H	" " "		12.5	99
I	Aqueous concentrate of rice polish		213 202 200 200 192 203	202 98
J	Wheat germ powder	Aqueous	27	
		80% CH ₃ OH	27.5	
K	Liver extract powder	Benzyl alcohol	12.5	
Average recovery of added thiamine chloride.....				101
" deviation				±3

* Corrected for the presence of interfering substances in the final test solution according to the method described in the text.

the case of Solutions G and H, the chloroform was first removed by *in vacuo* distillation. The 50 per cent ethyl alcohol extract (Sam-

ple F) was treated exactly in the same manner as the methanol solutions. The most significant column in Table III is that dealing with the percentage recovery of added thiamine chloride (100 micrograms added in each case). Theoretical values are consistently obtained. In the series reported the average recovery value is 101 per cent with an average deviation of ± 3 per cent.

Specificity of the Method for Chemical Determination of Thiamine in Biological Materials—When the thiamine and other substances in the KCl eluates from the zeolite filter are allowed to react with the reagent in the presence of phenol and ethyl alcohol, the colors developed are masked by that due to the pH indicator and to the phenolic derivative formed. Only the color resulting from the reaction between the vitamin and the diazonium salt is extracted by xylene. When the reaction is conducted in the presence of ethyl alcohol alone and use made of an outside pH indicator, other substances are found to be present which react with the reagent to yield pigments varying in color from pale yellow to orange. These compounds are not extracted by the xylene. In all of the tests reported in the present paper, and also in unpublished analyses of urine, aliquots of the KCl eluates were tested to determine whether the material responsible for the red color in the xylene layer is labile to alkali and heat as thiamine itself. When these solutions were adjusted to pH 13 and then heated in a water bath at 100° for 5 minutes, there was a decrease in intensity of the color extractable by xylene from 50 to 70 per cent of that initially present. Autoclaving these solutions at 15 pounds pressure for 16 hours at the same alkaline pH resulted in a complete disappearance of all color extractable by xylene. When the reaction was conducted in the presence of ethyl alcohol alone, the aqueous phase still showed the same colors, the intensities of which were comparable to those observed before the heat and alkali treatment. Control tests in which thiamine was added to other samples, treated similarly but now neutralized, indicated that the disappearance of color previously noted was due to a destruction of the compound responsible for the color and not to an inhibition of the reaction because of the production of interfering substances by the heat treatment.

In the present report no attempt was made to compare with the bioassay values the results obtained by chemical analyses.

In addition to the limitations of the biological assay (15) *only* free and not total thiamine (9) is determined by the method described, so that discrepancies should occur for this reason alone between the values obtained by these two methods. The chemical determination of phosphorylated thiamine, which is also biologically active (10), is discussed in Paper III (9).

SUMMARY

The chemical reaction between diazotized *p*-aminoacetophenone in alkaline solution and thiamine has been successfully applied to the determination of the thiamine concentration in extracts of rice polish, wheat germ, yeast, and liver. The method involves the quantitative extraction of the vitamin from biological materials, selective concentration of the vitamin by means of zeolite adsorption and subsequent elution, and estimation of the thiamine concentration by a modification of the chemical reaction allowing accurate analyses despite the presence of substances which formerly interfered with the reaction. The method is specific for free thiamine. The vitamin in the phosphorylated form is not determined.

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CHEMICAL DETERMINATION OF VITAMIN B₁

III. QUANTITATIVE ENZYMIC CONVERSION OF COCARBOXYLASE (THIAMINE PYROPHOSPHATE) TO THE FREE VITAMIN

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In Papers I and II (1, 2) the reaction between thiamine and diazotized *p*-aminoacetophenone in alkaline solution (3) was studied and a method of applying it to biological materials was described. Recently Lohmann and Schuster (4) reported that thiamine may exist in nature as the pyrophosphoric ester to constitute cocarboxylase, the coenzyme of carboxylase. Cocarboxylase has biological activity corresponding to free thiamine (4). It will be shown that in yeast as much as 75 per cent of the vitamin may be in the esterified form. Preliminary tests conducted with the phosphoric esters of thiamine¹ indicated that these compounds react with the diazonium salt to yield colored solutions (orange to pink). The pigments of such solutions, however, are not extracted by xylene, but remain in the aqueous phase.² Since in this chemical method the intensity of pigment in the xylene layer only is determined, the total thiamine content cannot be estimated unless a quantitative hydrolysis of the esterified forms

* Upjohn Fellow in Clinical Research, 1937-39.

¹ We are indebted to Dr. Joseph L. Melnick and Dr. Kurt G. Stern of the Laboratory of Physiological Chemistry, Yale University, for supplying us with synthetic preparations of these compounds. The cocarboxylase was in aqueous solution containing in addition some of the monophosphate and unchanged thiamine chloride. The monophosphate was supplied as the crystalline product.

² These colors cannot be observed unless the reaction is carried out in the presence of ethanol alone. When phenol also is used to increase the sensitivity of the reaction, these colors are masked by that due to the phenolic derivative.

to free thiamine is first carried out. This step is essential. A number of workers (5-7) have reported that, likewise, in the thiochrome method for determining thiamine, the cocarboxylase forms a thiochrome but that it is not extracted by isobutanol and hence cannot be estimated (6, 8).

Cocarboxylase may be hydrolyzed to thiamine monophosphate by 1 N HCl solution (4) but the latter is resistant to acid hydrolysis. Alkaline hydrolysis will completely remove the second phosphoric acid grouping but is impractical for analytical purposes because of concomitant destruction of thiamine. Kidney phosphatase (4, 7, 9) has been used as means for hydrolyzing the coenzyme to the free vitamin. However, with such a preparation the conversion has not been shown to be quantitative. Recently a more efficient procedure was reported by Kinnersley and Peters (10) who made use of a taka-phosphatase preparation for the hydrolysis of the esterified vitamin. The application of their method to quantitative analyses of biological materials for their total thiamine content was not attempted.

During the autolysis of yeast complete disappearance of cocarboxylase activity has been reported (11). Bacterial contamination likewise has been found to cause destruction of cocarboxylase (12). A complete hydrolysis of thiamine pyrophosphate (cocarboxylase) to free thiamine also would be associated with a disappearance of cocarboxylase activity. However, such a change was not demonstrated by either of these two investigators (11, 12), inasmuch as no attempts were made to identify any of the end-products resulting from the destruction of the coenzyme. In the present paper the quantitative enzymic conversion of phosphorylated thiamine to the free vitamin is described so that the method is applicable to the determination of the *total* thiamine content. By difference between the thiamine values, obtained before and after hydrolysis, the concentration of the vitamin in the phosphorylated form may also be estimated.³

EXPERIMENTAL

Tests with Pure Aqueous Solutions of Cocarboxylase and the Monophosphoric Ester of Thiamine—That the phosphoric esters of thiamine react with the diazonium salt to yield pigments which

³ A preliminary report of these studies has appeared elsewhere (13).

are not extracted by xylene has been described. These thiamine derivatives are adsorbed by zeolite but are not eluted by 25 per cent KCl or by 3.5 N H_2SO_4 . When solutions of these esters are passed through the zeolite filter and the filtrates and eluates subjected to analyses, no color reactions are obtained. That this finding is not due to the presence of interfering substances is shown by positive tests after the esters are added to these solutions. This is not in accord with the conception that zeolite operates only through the mechanism of base trading (7, 14). For this reason and because of observations previously described (2) we prefer to use the terms adsorption and elution in referring to the use of zeolite for the preparation of vitamin concentrates suitable for analyses.

Proof of Enzymic Conversion of Phosphorylated Thiamine to the Free Vitamin during Autolysis of Yeast—In Paper II analyses of a number of yeast preparations were reported (see Table III (2)). One sample (G) which had been prepared⁴ under careful control gave values which were 27 per cent of that found by biological assay. In the course of the preparation of Extract H the aqueous yeast suspension had been exposed to the atmosphere with no preservative added for a period of approximately 24 hours. In this case good agreement with the biological assay value was obtained. We have investigated the mechanism of this increase in the content of free thiamine.

In aqueous extracts prepared at 70° from a dried yeast powder,⁵ as previously described (2), 40 micrograms of thiamine per gm. of the original yeast powder were found. When an aliquot of the extract was subjected to bacterial activity for a period of 48 hours, no change in the value for the thiamine content was observed. This was also true when 100 micrograms of the crystalline thiamine monophosphate were added to the extract and similarly exposed to bacterial contamination.

⁴ These aqueous yeast extracts were prepared by Dr. Harold Levine of the Premier-Pabst Corporation, Milwaukee, and submitted to us for chemical analysis. The authors are indebted to Dr. Levine for a number of helpful suggestions.

⁵ A generous amount of this hot air-dried yeast powder, used as a source of phosphatase, was supplied by the Premier-Pabst Corporation, Milwaukee. This product is the same as Sample A, described in Table III of Paper II (2).

On the other hand, when a suspension of the yeast powder, with or without toluene added as a bacterial inhibitor, was allowed to stand 2 days at room temperature, values of 162 micrograms of thiamine were obtained. Biological assay of this yeast had indicated a potency of 56.5 international units. No increase in the values of free thiamine occurred, on standing at room temperature, in yeast suspensions which had first been heated to 70° for 30 minutes or which contained added chloroform or in aqueous solutions prepared from 80 per cent methyl alcohol extracts.

Enzymes are believed to be protein in character or associated with a protein carrier. The low values for thiamine were obtained after the use of procedures by which the enzyme was precipitated or inactivated before opportunity for hydrolysis was permitted. These findings indicate that the change in the thiamine values is due to an enzymic hydrolysis of the phosphorylated vitamin to free thiamine.

The enzyme present in yeast is capable of hydrolyzing quantitatively added phosphorylated thiamine. Theoretical values for extra free thiamine were obtained after the crystalline monophosphate was incubated with the yeast suspension. Likewise, after the incubation of the yeast powder with protein and enzyme-free aqueous extracts of the same powder, all of the vitamin of both yeast powder and extracts was determined as free thiamine.

Optimal Conditions for Enzymic Hydrolysis of Phosphoric Esters of Thiamine. *Optimal pH*—To each of five flasks containing 30 cc. of water, 0.2 cc. of toluene, and 100 micrograms of crystalline thiamine monophosphate, 0.5 gm. of the yeast powder was added. The pH of each suspension was adjusted by adding various quantities of 1 N H₂SO₄ or NaOH solutions. The solutions were sufficiently buffered in themselves so that the initial pH values were maintained throughout the incubation period. The hydrolysis was allowed to proceed for a period of 24 hours at 37.5°. The results of the analyses are summarized in Table I. These tests indicate that the pH optimal for the hydrolysis of phosphorylated thiamine is rather closely restricted to that of 4.5. The low value obtained when the reaction was allowed to proceed at pH 8 may be due in part to the instability of the vitamin in alkaline solution.

Time Required for Completion of Enzymic Hydrolysis—The tests

described above were repeated but this time all the suspensions were at pH 4.5, with the duration of the incubation periods allowed to vary. The results, summarized in Table II, indicate that the reaction is completed by the 12th hour. Plotting the course of the reaction in terms of percentage hydrolysis *versus* time gives a curve characteristic of enzymic reactions.

Optimal Temperature for Enzymic Hydrolysis—The above tests were repeated with the pH adjusted in each case to 4.5 but with the incubation temperature varying from 10° to 70°. The reactions were allowed to continue for a period of only 1 hour.

TABLE I
*Optimal pH for Enzymic Hydrolysis of Phosphoric Esters of Thiamine**

pH	Total thiamine chloride found	Thiamine liberated by hydrolysis†	Percentage hydrolysis of thiamine ester†
	<i>micrograms</i>	<i>micrograms</i>	
1	20	0	0
2.5	87	67	46
4.5	168	148	102
6	150	130	90
8	58	38	26

* The aqueous yeast suspensions were incubated at 37.5° for a period of 24 hours.

† The 0.5 gm. of yeast powder used in each test had a total vitamin content equivalent to 85 micrograms of thiamine chloride. 20 micrograms existed initially as free thiamine. The 100 micrograms of thiamine monophosphate, which were added to each mixture, are equal to 80 micrograms of the free vitamin (4) so that a total of 145 micrograms of thiamine existed in the esterified forms.

It is apparent from Table III that an incubation temperature of approximately 45° is optimal for the hydrolysis of the phosphoric esters of thiamine. At 70° no hydrolysis is possible owing to the inactivation of the enzyme by heat.

Attempts to Isolate Phosphatase—Because of the indications of the protein nature of the enzyme, attempts were made to isolate from the active yeast powder a soluble protein fraction free from thiamine that would exhibit enzymic activity. Two different procedures were used. The protein in the aqueous extract⁶ of the yeast powder was precipitated by 80 per cent methyl alcohol at a

⁶ Prepared at room temperature.

temperature of approximately 5°. The precipitate was dehydrated by consecutive washings with chilled absolute alcohol and ether and finally dried at 50°. In the other procedure the protein

TABLE II
*Time Required for Complete Enzymic Hydrolysis of Phosphoric Esters of Thiamine**

Incubation time	Total thiamine chloride found	Thiamine liberated by hydrolysis†	Percentage hydrolysis of thiamine esters†
hrs.	micrograms	micrograms	
0	20	0	0
1	88	68	47
3	137	117	81
6	147	127	88
12	160	140	97
18	167	147	101
24	168	148	102
30	163	143	99

* The aqueous yeast suspensions at pH 4.5 were incubated at 37.5°.

† The same explanatory statements added as a foot-note to these columns in Table I apply here also.

TABLE III
*Optimal Temperature for Enzymic Hydrolysis of Phosphoric Esters of Thiamine**

Incubation temperature	Total thiamine chloride found	Thiamine liberated by hydrolysis†	Percentage hydrolysis of thiamine esters†
°C.	micrograms	micrograms	
10	62	42	29
25	83	63	43
37.5	93	73	50
45	110	90	62
55	97	77	53
70	20	0	0

* The aqueous yeast suspensions at pH 4.5 were incubated for a period of 1 hour at the temperatures indicated.

† The same explanatory statements added as a foot-note to these columns in Table I apply here also.

fraction was obtained by dialyzing⁷ the aqueous extract against running water, concentrating the remaining solution *in vacuo*

⁷ Cellophane No. 300, kindly furnished by E. I. du Pont de Nemours and Company, Wilmington, was used as the semipermeable membrane. It is impermeable to protein and therefore suitable for the dialysis of protein solutions (15, 16).

(35–40°) to a syrup, and dehydrating the material in a vacuum desiccator. In both cases approximately 65 mg. of the ground final product were equal to 1 gm. of the original yeast preparation. Despite the precautions noted above, the protein present in the extract powders was largely denatured. However, a filtrate still gave a positive biuret test, a precipitate with sulfosalicylic acid, and a slight but positive heat coagulum. The “enzyme” powders were found to be free from thiamine and incubation of them with the crystalline monophosphate resulted in a 30 to 50 per cent hydrolysis of the ester. Control tests showed no conversion of the ester to free vitamin in solutions similarly treated but without the enzyme powders. These results indicate that the enzyme activity is associated with the water-soluble protein fraction of the original yeast powder. Inasmuch as quantitative hydrolysis of phosphorylated thiamine did not occur in the artificial systems set up to test the enzyme powders, we have continued to use the active whole yeast preparation as the source of the phosphatase.

Application of Yeast Phosphatase to Determination of Total Thiamine Content in Biological Materials and to Estimation of Amount in Phosphorylated Forms—In Table IV we have presented some of our studies in which yeast phosphatase was used to convert all of the vitamin B₁ present to free thiamine. By difference between the thiamine values, obtained before and after hydrolysis, the concentration of the vitamin in the phosphorylated form was determined. Samples A and B were found to contain soluble protein. Incubation of aqueous suspensions of these yeast powders under optimal conditions for a period of 24 hours resulted in constant yields of free thiamine, which approximated the values obtained by biological assay. Sample C was found to contain no soluble protein and was unable to hydrolyze the phosphorylated vitamin present. However, the incubation of either yeast powder Sample A or B with an aqueous suspension of Sample C resulted in hydrolysis of its esterified vitamin.⁶ In similar fashion the phosphorylated thiamine present in Samples D, G, and L was converted to free thiamine. Analysis of Sample M, an 80 per cent methanol extract of Sample B, indicates that this solvent is capable of ex-

⁶ For this purpose 0.5 gm. of the active yeast powder was used. The thiamine content of Sample C was obtained by difference between the total thiamine recovered and that present in the yeast powder furnishing the enzyme.

TABLE IV

Application of Yeast Phosphatase to Determination of Total Thiamine Content of Biological Materials and to Estimation of Amount in Phosphorylated Forms

Sample*	Description of preparation	Chemical assay values			Biological assay value†
		Free thiamine	Phosphorylated thiamine	Total thiamine	
		micrograms per gm.	micrograms per gm.	micrograms per gm.	micrograms per gm.
A	Hot air-dried yeast powder	40	130	170	169
B	" " " "	37	121	158	180
C	Dried yeast powder, free from soluble protein	45	39	84	About 120
D	Protein-free, artificially sweetened, concentrated aqueous extract of yeast	30	43	73	59‡
G	Protein-free, aqueous extract of yeast powder with CHCl ₃ as preservative	3.9	11.6	15.5	17.4
L	Protein-free, aqueous extract of yeast powder§	45	34	79	About 120
M	Protein-free, aqueous solution prepared from initial 80% CH ₃ OH extract of yeast powder§	38	126	164	180
I	Protein-free, aqueous concentrate of rice polish	203	42	245	259
J	Wheat germ powder	27.5	3.5	31	18-58
J'	Protein-free, aqueous extract of powdered wheat germ§	27.5	3	30.5	18-53

* The same preparations as are described in Paper II (2).

† The biological assay values were converted into micrograms of thiamine chloride, assuming that 3 micrograms of thiamine chloride are equal to 1 international unit.

‡ This value was not obtained by any of the special assays conducted as part of this study but was that indicated on the label of the preparation.

§ The values obtained with each of these solutions are expressed in terms of micrograms of thiamine chloride per gm. of original dry material.

|| Biological assays reported in the literature (17) indicate values ranging from 18 to 58 micrograms of thiamine chloride per gm. of wheat germ.

tracting quantitatively both forms of thiamine from biological materials.⁹ Incubation of extracts of rice polish (Sample I) or of wheat germ (Sample J') with the active whole yeast powder results in recoveries of additional amounts of thiamine. Although accurate analyses were obtained by the addition of whole wheat germ (Sample J) to the aqueous yeast suspension, this may not be possible under the above conditions of experimentation when assays of foods containing intact cells are attempted. In such tests contact between enzyme and substrate would be limited by the rate of diffusion of the phosphorylated vitamin through the cell wall. For such assays it seems preferable to add the active yeast powder to *extracts* of the material to be tested.

Yeast is the best known source of the coenzyme, cocarboxylase, and analyses indicate that as much as 75 per cent of the total thiamine may be present in the phosphorylated form. More of the vitamin may have been originally present as these esters, since the ratio of phosphorylated to free thiamine in the final product will be dependent on the extent to which the conditions of preparation favor phosphatase activity.

SUMMARY

By the chemical method, based upon the reaction between thiamine and diazotized *p*-aminoacetophenone, it is not possible to determine either of the phosphorylated thiamines (cocarboxylase or the monophosphate), until they are hydrolyzed to the free vitamin. In yeast preparations this is accomplished by the action of a phosphatase which is liberated from the cell during the drying of the yeast. The optimal conditions for the enzymic hydrolysis were determined. The enzyme activity was found to be associated with a water-soluble protein fraction in the yeast preparation. The incubation of an active yeast powder with a number of thiamine concentrates, such as extracts of yeast, rice polish, and wheat germ, results in a quantitative conversion of

⁹ This finding is confirmed by the rat growth data obtained by Dr. Harold Levine of the Premier-Pabst Corporation, Milwaukee, demonstrating that all of the thiamine is removed quantitatively from dried yeast powder by a procedure of extraction with 80 per cent methanol as well as by an aqueous extraction method similar to those used in the present studies.

all the vitamin present into the free form which may be determined chemically. By difference between thiamine values, obtained before and after hydrolysis, the concentration of the phosphorylated vitamin may be estimated. Dried yeast may contain as much as 75 per cent of its thiamine in the esterified form, while in rice polish and wheat germ the major portion exists as the free vitamin.

We are indebted to the Directors of the Research Laboratories of The Upjohn Company, Kalamazoo, the Premier-Pabst Corporation, Milwaukee, and Burroughs Wellcome and Company, Tuckahoe, for conducting a number of special biological assays of preparations used in the present study.

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THE DISAPPEARANCE OF AMINO NITROGEN FROM CELL-FREE LIVER EXTRACTS. II

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The possibility of preparing a cell-free liver extract having constant and reproducible properties with respect to the "disappearance" of amino nitrogen (1) has been further investigated. Our results, obtained with homogeneous cell extracts, can of course not be considered applicable to reactions in living cells, in which velocities are largely determined by heterogeneous conditions. The isolation of physiologically linked units in the chains of cell reactions is necessarily a difficult undertaking. The fact, however, that the reaction which we have called "disappearance of amino nitrogen" occurs consistently under various conditions seems to form a promising basis for its closer investigation.

Methods

Method I, described in our first paper (1), is reproducible but not suitable for further purification. Other methods, investigated in continuation of our studies, showed a marked tendency to yield extracts extremely low in amino nitrogen. This was caused by the acceleration of the disappearance of amino nitrogen when the solutions were heated. We shall refer to the disappearance reaction as the *D* value, *D* activity, or *D* reaction. *D* is always given as the percentage of the preceding value, the time and temperature being indicated. The concentration corresponds to 50 gm. of solids in 100 gm. of solution after the original pH level of 5 had been adjusted to 7.4, which was maintained during all experiments. This was the standard solution. All *D* values are corrected for the small dilutions incident to maintenance of this pH.

The *D* reaction in fresh standard solution was generally about 10 (sometimes rising to about 20), as determined at 40° in 24 hours (D_{40}^{24}). Dialysates from the standard solution, which were evaporated at 14°, gave considerably higher values.

In almost all experiments any proteolytic activity was determined by the methods of Linderström-Lang and Holter (2); the substrates were casein (pH 3.8 and 7.8), alanylglycine (pH 7.4), and chloroacetyltyrosine (pH 7.4).

In several preparations an active, comparatively stable, dry powder was obtained. A 25 kilo quantity of fresh beef liver was freed from superficial fat, carefully ground, mixed with half its weight of water, and adjusted to pH 5 by the gradual addition of 60 per cent sulfuric acid with efficient stirring during 1.5 hours, the pH being measured with the glass electrode. Acetone was added to make a final amount of 5 liters in 10 liters of the whole mixture. This was stirred for 24 hours at room temperature, and the acetone (about half of which usually evaporated during the extraction) made up to the original concentration. The solids were then removed (1) and the clear solution evaporated at 16° to 5 liters. An equal volume of acetone was added in portions with violent shaking, the mixture was held at 2° overnight, and the flocculent, somewhat oily precipitate was filtered off at the same temperature. The clear filtrate was evaporated at 16° to 1 liter, and held overnight at -15°-16°; a yellow voluminous precipitate was removed by rapid centrifugation, and tricresol was added (1). In early experiments this solution was stored at -20° in small bottles; later, in order to avoid the danger of acid fermentation and of a *D* reaction which probably occurred during unavoidable warming to room temperature, the solutions were freed from water by repeatedly pouring 200 cc. portions into 1 liter quantities of 2:1 absolute alcohol-ether. The oily precipitates were converted to semisolid form by repeated energetic agitation with more alcohol-ether. This procedure took 15 minutes for each portion and was completed by washing with petroleum ether and evaporation of a thin layer in a vacuum desiccator over phosphorus pentoxide, the desiccating agent being renewed twice daily. Determinations of the dry weight (Pregl) of the porous product showed a loss of 1.08 per cent after 24 hours and 0.05 per cent after 1 week. This preparation dissolved readily in water and displayed marked buffering power.

Two such preparations (from 50 kilos of liver in all) were united; the D_{410}^{24} value remained fairly constant during 3 months.

EXPERIMENTAL

The dry preparation, which contained about 60 per cent of glucose, was made up to a 30 to 40 per cent solution of pH 5. This was decolorized by two filtrations through a 2 cm. layer of activated carbon, which adsorbed about 2 per cent of the dry weight. The D_{410}^{24} value was not markedly affected ($D = 8.4$). The color reappeared after 48 hours at pH 7.4 and 40°. In Table I is presented a comparison of this filtrate, a 20 per cent glucose solution, both of which were held at pH 7.4 and 40° during 48 hours, and a newly prepared standard solution.

TABLE I

Extinction Coefficients of Yellow Color Appearing after 48 Hours at 40°

Wave-lengths	Filtrate	Glucose solution	Standard solution
Å.			
4300	0.62	0.81	0.66
4700	0.34	0.50	0.38
5000	0.18	0.22	0.21
5300	0.08	0.12	0.12
5700	0.06	0.05	0.05

No flavins were present in the standard, as was demonstrated by the absence of lumiflavin after irradiation with ultraviolet light from a high pressure mercury lamp for 2 hours at 10 cm. distance. The standard solution contained no bile pigments, but bile acids, a small amount of urea, 1.25 mg. of ammonia per cc., and 20.2 mg. of total nitrogen per cc. were present. Arnold's reaction (by saturation with ammonium sulfate) was strongly positive. The standard solution always gave a very faint opalescence on addition of 10 per cent of trichloroacetic acid or sulfosalicylic acid; such mixtures showed a faint precipitation after 24 hours. The dialysates never showed more than a barely appreciable opalescence on treatment with these reagents.

A standard solution and its dialysate were hydrolyzed by heating at 100° with hydrochloric acid (20 gm. per 100 cc. of solution in an evacuated tube). In Table II are shown the results of one

such experiment, in the interpretation of which due allowance must be made for the presence of large amounts of sugar. Loss of nitrogen occurred by adsorption on the large amounts of humin formed during hydrolysis. In the standard solution the amino nitrogen value was not altered by hydrolysis, but increased in the dialysate. A discussion is deferred for the present. The percentage ratios of amino nitrogen to total nitrogen before hydrolysis were 48.4 and 20.5 in the dialyzable and non-dialyzable fractions respectively. This demonstrates the presence of a very small amount of a non-dialyzing nitrogenous substance and agrees with the comparatively higher increase of amino nitrogen after hydrolysis of the non-dialyzable part (50 per cent) compared to the dialyzable part (40 per cent).

TABLE II

Nitrogen Distribution in Standard and Dialyzed Solutions after Dialysis through Collodion Membranes Accelerated by 0.2 Atmosphere Pressure during 24 Hours

The values are given in mg. per cc.

Sample	Total N before hydrolysis	Total N after hydrolysis	Amino N before hydrolysis	Amino N after hydrolysis
Standard solution.....	18.13	15.30	11.9	11.3
Dialyzed "	21.30	18.0	10.3	14.5
Non-dialyzable solution.....	0.28	0.12	0.058	0.088

The solution and dialysate were hydrolyzed 20 hours.

The question of the presence of real proteins, specific or inert, in the standard solution is, however, not satisfactorily answered by the above experiments. In accordance with prevailing views of a "synthesis" involving amino acids a separation from the substrate of enzymes which might have been present was continued by means of dialysis. The working hypothesis involved the possibility of inactivating the *D* reaction if the enzymes were non-dialyzable under various conditions. Collodion tubes prepared according to Sørensen's technique were employed and rapid dialysis was secured by the use of stirring and air pressure of 0.2 to 0.25 atmosphere. After dialysis of 800 cc. of standard solution for 24 hours the inside solution, when concentrated to 85 cc., contained 0.088 mg. of amino nitrogen per cc., whereas the outside solution, after concentration to 375 cc., contained 12.58 mg. of

amino nitrogen per cc. In Table III is shown an unusually high *D* reaction. In this experiment a loss of 2.1 per cent of total nitrogen occurred, the whole of this being found as ammonia. This was the only case in which the formation of ammonia was encountered; in other instances no ammonia escaped even at 100° and pH 7.4. Table III also demonstrates the readily reproducible *inhibiting* influence of the inside solutions on the *D* reaction.

Repeated experiments with dialysis have always indicated that all but a small fraction of the solids in the standard solution are dialyzable. After a *D* reaction both standard solution and dialysate consistently yielded bulky precipitates on saturation with ammonium sulfate and on treatment with trichloroacetic or sulfo-salicylic acid. These precipitates were especially pronounced

TABLE III
Inhibiting Effect of Non-Dialyzable Fraction on D_{40} Reaction of Dialyzed Standard Solution

Sample	Time	Total N	Amino N	<i>D</i> reaction	Loss in NH ₃ -N
	hrs.	mg. per cc.	mg. per cc.		mg. per cc.
Concentrated dialyzed solution	0	19.76	11.25	$D_{40}^{19.5} = 76$	0.41
	19.5	19.31	2.70		
Dialyzed solution mixed with concentrated non-dialyzable solution	0	17.86	8.21	$D_{40}^{24} = 30.6$	0
	24	17.78	5.70		

after the *D* reactions had occurred in dialysates. As stated above, the D_{40}^{24} reaction consistently occurs, even after 3 months of storage; this was not the case with older preparations. Although little can be said about the products formed in the fresh or in the older preparations, it is certain that even in those experiments in which no precipitates were produced, only a part of the products formed by the *D* reactions is dialyzable.

In Table IV is reported an experiment in which an old standard solution was dialyzed by means of suction through a very thin cellulose acetate membrane. This membrane had been applied to the outside of an alundum cylinder¹ in the form of a solution in glacial acetic acid. The coated cylinder was immersed in the

¹ The authors are deeply indebted to Dr. A. Tiselius for the alundum cylinder and the cellulose acetate solution.

solution and, when the velocity of dialysis decreased, the solution was diluted to its original volume with water; this dilution was repeated eight times. The entire dialysate was heated to 40° and again dialyzed. All the amino nitrogen remaining after the D_{40}^{24} reaction passed through the membrane, whereas 12.5 per cent of the total nitrogen had been rendered non-dialyzable.

In order to decide whether the D reaction was influenced by anaerobic conditions, a standard solution was freed from air by expiration of nitrogen for 1 hour, and all precautions were sub-

TABLE IV

Formation of Non-Dialyzable, Nitrogen-Containing Substance by D_{40} Reaction in Dialyzed Solution

The values are given in mg. per cc.

Sample	Total N	Directly determinable amino N	Amino N after hydrolysis
First dialysate.....	14.15	6.89	9.50
Same after D reaction ($D_{40}^{120} = 34.4$).....	14.21	4.52	
Twice dialyzed solution.....	12.48	4.43	7.93

TABLE V

D_{40} Reaction in Standard Solution under Anaerobic Conditions

Standard solution	Time	D_{40}^{24} reaction	Amino N	Total N
	<i>hrs.</i>		<i>mg. per cc.</i>	<i>mg. per cc.</i>
In nitrogen	0		6.80	20.60
	24	21.9	5.31	20.53
In air	0		7.40	20.60
	24	17.7	6.09	20.60

sequently taken to exclude oxygen. The results of this experiment, together with those of a control in which air was employed, are shown in Table V. No loss of ammonia occurred. The most conspicuous result is the slightly higher D reaction observed in the absence of oxygen. This result is not in accordance with the findings of Voegtlin *et al.* (3) which, however, were obtained in the presence of liver cells. We wish merely to state that *the velocity of the D reaction under the conditions of our experiment seems to be independent of the presence of oxygen.*

An outstanding feature, briefly mentioned in an earlier paper

(1), is the influence of temperature on the rate of the *D* reaction. The stability of the *D* reaction at 100° was tested by heating a series of standard solutions in a water bath for periods of 2 to 60 minutes. The results are shown in Table VI. In order to obtain a rapid increase in temperature small volumes of solution were used and the heating was initiated over an open flame (43 seconds to reach 100°). During the heating the volumes were held constant and there was no loss of ammonia. Amino nitrogen was determined immediately after the heating, and the solutions were then kept at 40° for longer periods. The solution before heating contained 8.36 mg. of amino nitrogen per cc. *D* reactions displayed little difference after 30 and 60 minutes boiling time respectively, and tended to reach a limiting value. This effect is obviously due to the inhibition of some process concerned

TABLE VI
Stability of D₄₀ Reactions in Standard Solutions Heated at 100° for Varying Times

	Boiling period					
	2 min.	4 min.	6 min.	8 min.	30 min.	60 min.
<i>D</i> reaction immediately after boiling.....	9	20	21	41	51	59
<i>D</i> ₄₀ reaction after 24 hrs.	17	21	23	25	0	0

in the *D* reaction. According to the *D*₄₀ reactions, this inhibition is practically complete after 30 minutes boiling. However, the thermostability of the *D* reaction is surprisingly high, for only a small loss of activity occurred after 8 minutes of boiling.

In a second series the standard solutions were heated for 30 minutes at temperatures between 40° and 100° and thereafter held at 40°. The first line in Table VII indicates that inhibition is almost complete after 30 minutes at 100°, as is also indicated in Table VI. On the other hand, little or no inhibition occurred during 30 minutes at 80°.

Potassium cyanide in a 0.01 *N* concentration increases the rate of the *D* reaction, as is shown in Table VIII, which likewise demonstrates the influence of hydrogen ion concentration. The complete inhibition of the *D* reaction at pH 5 was relieved by the presence of cyanide and a marked stimulating effect of the reac-

tion at pH 7.4 observed. This effect is particularly noticeable with the dialysate. In most experiments the D_{40} reaction was followed during several days and pursued a linear course for about 72 hours, generally ceasing when the amino nitrogen values had fallen to a level of about 2 mg. per cc. The addition of 0.01 N KCN at pH 7.4 to "exhausted" solutions which had shown no change in amino nitrogen during 48 hours at 40° always brought about a D_{40}^{24} reaction of about 10. We consider that this phenomenon demonstrates a reactivation by cyanide in concordance with the stimulating effect shown in Table VIII.

TABLE VII
Stability of D_{40} Reactions in Standard Solutions Heated at Different Temperatures for 30 Minutes

	Heating temperatures			
	40°	60°	80°	100°
D reaction immediately after heating period..	0	5	23	55
D_{40} " after 24 hrs.	17	15	15	2

TABLE VIII
Stimulating Effect of KCN (0.01 N) on pH Stability of D Reaction

	D_{40}^{24} reaction		
	pH 5	pH 7	pH 8
Standard control.....	0	18	16
" " + KCN.....	10	25	
Dialysate of standard.....		5	
" " " + KCN.....		13.3	

At pH 7 iodoacetic acid, 2 per cent sodium fluoride, 0.008 N cupric chloride, and H_2S (to saturation) exercised no inhibitory influence. The D activity was not influenced by adsorption with carbon. Precipitation with lead acetate at pH 7.4 removed about 10 per cent of the solids; the D_{40}^{24} activity in the lead-free filtrate varied from 0 to 9 in four experiments. Extensive attempts to differentiate the system by extraction with mixtures of acetone, ethyl alcohol, and propylene glycol have as yet led only to inactivation or to the production of fractions which occasionally displayed a "reactivation" when reunited. Attempts will be made

to remove the bulk of the sugar by electrophoresis. Standard solutions and dialysates gave a strong Arnold reaction, but it has not been found possible to relate this to the *D* reactions. In very few cases was a proteolytic activity found. The system concerned with the *D* reactions can be prepared in various ways, all of which lead to preparations containing blood-generating activity and others active towards pernicious anemia. This phase of the subject will be further explored.

DISCUSSION

The system concerned with the disappearance of amino nitrogen has been further purified. Considering the state of impurity, the reaction proceeds fairly accurately as a first order reaction. From dry material an active colorless solution can be prepared by means of adsorption on carbon.

The factors participating in the reaction can be dialyzed through membranes known to be impermeable to molecules greater than 10,000. It seems unlikely that a reversibly acting enzyme of high molecular weight is involved. The heat stability of the reaction is markedly higher than that of many proteolytic enzymes, the presence of which has not consistently been detected in our preparations. The presence of other types of intracellular enzymes is not excluded. Of these very little is known. The milieu might effect an even more determining influence than has been proved. We are not in the position to relate our results with those of Bergmann (4-6) with proteolytic enzymes. The reaction reported in this paper might belong to the group commonly termed catalyzed processes. The fact that the active system is dialyzable does not necessarily preclude the presence of active enzymes. The unusually high heat stability is to be regarded merely as an observation and not as a conclusive disproof of the enzymatic character of the reaction. Discussion of the stimulating effect of cyanide must be deferred until our system has been further purified.

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A SIMPLE CATAPHORESIS APPARATUS

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(Received for publication, October 7, 1938)

The cataphoresis apparatus described here was designed to provide a cheap apparatus capable of almost quantitative separations of proteins with isoelectric points quite close together (serum albumin and globulin) and giving large yields of purified end-products with little dilution. It has proved of value in two separations and would seem to be applicable to a wide number of problems. Sulfhemoglobin and oxyhemoglobin together have been separated from the colloidal reaction mixture in which the former is made, and serum albumin has been separated from 2.00 cc. samples of plasma.

The apparatus (Fig. 1) consists of a straight, water-jacketed glass tube with reservoirs at top and bottom to insure adequate buffering against changes in pH arising at the Ag-AgCl electrodes (1). The straight tube has the advantage over a U-tube that all portions of the solution placed in it migrate in the same straight line, none being on the inside track of a curve.¹

The use of ground glass wool² as a filler minimizes convection currents and provides a means of layering the sample and of

¹ A simple push-pull amplifier, made from inexpensive radio material at a cost of less than \$25 including meter, gave 200 milliamperes at 750 volts very satisfactorily. With the dimensions of the apparatus as given, however, heating sufficient to distort the boundaries is apt to occur with currents over 100 milliamperes.

² Ground glass wool was prepared by grinding glass wool (Merck) with relatively small amounts of clean sand and water, separating the product by stirring in large beakers with water, and decanting so as to obtain fairly homogeneous particles settling within about 10 seconds in a liter beaker. Matting is avoided by drying with alcohol and ether at room temperature in a thin layer and grinding lightly in a mortar before use.

collecting the material which has migrated a certain distance without disturbing the remaining solution. Good layering is aided by adding starch or sucrose to the buffer in the lower buffer reservoir.

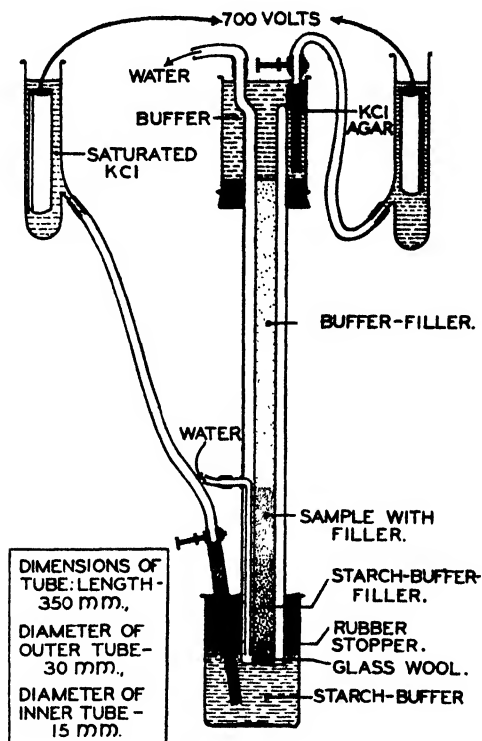


FIG. 1. Cataphoresis apparatus

Operation

The lower reservoir is filled with buffer and the water-jacketed tube inserted; the cooling water is turned on; a plug of cotton is rammed down to the bottom of the tube; and filler is dropped in to give a section a few cm. in length; the tube is tapped and the filler tamped down. The sample is inserted, followed by filler until only a trace of sample remains above it. Any air bubbles are removed by tapping. Buffer and filler are added and tapped and tamped down every few cm. until the desired height is reached.

The remainder of the tube and the top reservoir are filled with buffer, the electrode inserted, and the current turned on.

As the cataphoresis progresses, the electrodes must be exchanged occasionally to avoid exhausting the AgCl coating. Samples may be withdrawn and the buffer above the filler renewed as often as necessary.

Results

Several mixtures prepared from 20 per cent hemoglobin solution by the addition of H_2S and H_2O_2 followed by dialysis were subjected to cataphoresis at pH 8.0 for 5 to 6 hours. The total pigment recovered was at most 70 per cent of the amount in the sample, was free of non-sulfhemoglobin sulfur, and was a mixture of oxyhemoglobin and sulfhemoglobin, both components having the same rate of migration (2).

Three 2.00 cc. samples of oxalated human plasma at pH 8.0 were subjected to cataphoresis between 6 and 12 hours at constant voltages between 200 and 500 volts. The volume of the sample removed was 4.00 to 5.00 cc., a dilution of about 2-fold.

Experiment	Duration	Albumin recovered	Albumin-globulin ratio of product
	hrs.	per cent	
A	6	43	∞
B	6	66	18:1
C	11	93	

SUMMARY

A simple cataphoresis apparatus is described, capable of subjecting small (and presumably, with slight change in design, large) samples of protein solution to quantitative cataphoresis, with good separation on the basis of rate of migration, easy removal of samples, and only slight dilution.

The application of the apparatus to the separation of sulfhemoglobin-oxyhemoglobin mixtures and of serum albumin is described.

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STUDIES ON OCTOPINE

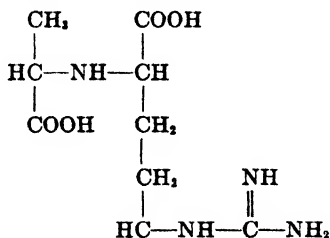
I. THE SYNTHESIS AND TITRATION CURVE OF OCTOPINE*

By J. LOGAN IRVIN AND D. WRIGHT WILSON

(From the Department of Physiological Chemistry, School of Medicine,
University of Pennsylvania, Philadelphia)

(Received for publication, November 5, 1938)

In 1934 Moore and Wilson (9, 10) reported the isolation from scallop muscle of a crystalline material which was proved to be octopine, a compound previously isolated by Morizawa from octopus muscle (11). The structure was studied by Moore and Wilson, who concluded that octopine is arginine, the α -amino group of which is attached to the α -carbon atom of propionic acid. It may be given the name N^{α} -(1-carboxyethyl) arginine. Akasi (2) arrived at the same conclusion regarding the structure of



octopine and was the first to synthesize it. He also determined its optical configuration. The partially inactive compound was independently synthesized by ourselves (8). Ackermann and

* This and the following two papers are taken from the thesis presented by J. Logan Irvin to the Faculty of the Graduate School of the University of Pennsylvania in 1938 in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Preliminary reports of this work have been presented in the *Proceedings of the Society for Experimental Biology and Medicine*, April, 1937 (8), and before the Physiological Society of Philadelphia, April 19, 1937, and the American Society of Biological Chemists at Baltimore, April 2, 1938 (7).

Mohr (1) have recently repeated Akasi's synthesis. After we had seen the papers of Akasi, we decided to repeat our synthesis, using a lower temperature to diminish racemization and employing his method of separating the natural isomer from the unnatural enantiomorph by means of the difference in the solubility of the picrates. Our synthesis is described in this paper.

The titration curve of octopine has been determined with a glass electrode. This curve furnishes additional evidence for the two acidic and two basic groups of octopine.

EXPERIMENTAL

Synthesis of Octopine—In our first paper (8) the synthesis of a partially optically inactive octopine was described. As data for the complete elementary analysis of the compound and its picrate were not given in that paper, they are presented below.

$C_9H_{18}N_4O_4$.	Calculated.	C 43.92, H 7.37, N 22.76
	Found.	" 43.87, " 7.04, " 22.49
$C_9H_{18}N_4O_4 \cdot C_6H_5N_3O_7$.	Calculated.	C 37.88, H 4.45, N 20.63
	Found.	" 38.35, " 4.50, " 20.24

Our final method of synthesis was as follows: A reaction solution containing 2.0 gm. of *d*-arginine methyl ester dihydrochloride, 1.4 gm. of *dl*- α -bromopropionic acid ethyl ester, 2 equivalents of sodium ethylate, and a small amount of potassium iodide in 10 cc. of absolute ethyl alcohol was refluxed at 80°. Another equivalent of sodium ethylate was added in small portions at hourly intervals during the early part of the period of reaction which was continued for 12 hours. The solution was then made acid with sulfuric acid, and after the removal of iodide, chloride, and bromide with silver sulfate, the filtrate was heated gently on the water bath for 1 hour in order to hydrolyze the esters. Analysis of a sample of this solution indicated a 92 per cent decrease in amino nitrogen, as determined by the Van Slyke method. An analysis for guanidine groups by the Sakaguchi method as previously employed (10) showed about 117 per cent of the value expected from the amount of arginine used in the reaction. Since octopine does not contain a free amino group and gives with the Sakaguchi analysis a value 20 per cent higher than that given by an equivalent of arginine, these analyses indicated a fairly complete synthesis of octopine.

The solution was then treated with silver nitrate and barium hydroxide for the precipitation of octopine and unchanged arginine. The silver-baryta precipitate was decomposed in the usual manner. The solution was made very faintly acid to Congo red with sulfuric acid and was treated at the boiling point with 0.22 gm. of flavianic acid. After standing overnight a small amount of the typical plates of arginine flavianate settled to the bottom of the beaker. The flavianate was removed by filtration.

After extraction of the excess flavianic acid from the filtrate in the usual manner, the solution was freed of sulfate and evaporated under reduced pressure to a small volume. By the addition of a saturated solution of picric acid, small clustered needles resembling octopine picrate separated from the solution. A yield of 1.39 gm. of picrate was obtained; this was 76.6 per cent of the theoretical for one isomer, based on the amount of arginine methyl ester dihydrochloride used in the reaction. The filtrate contained the picrate of the unnatural enantiomorph of octopine. After several recrystallizations of the picrate of the natural isomer, the material melted with decomposition at 226° .¹ A mixture with a known sample of octopine picrate showed no depression. The picrate of the synthetic compound when analyzed by our Sakaguchi procedure gave 97 per cent of the expected value when octopine was used as a standard. The color given by the picrate with these reagents was identical with the characteristic purple color given by octopine, thus differing from the orange-red tint produced by arginine. The picrate contained no water of crystallization and no free amino nitrogen and gave the following analyses.

$C_9H_{18}N_4O_4 \cdot C_6H_3N_3O_7$.	Calculated.	N 20.63,	picric acid 48.2
	Found.	" 20.50,	" " 48.1

Picric acid was determined by the method of Cope and Barab (4).

The free synthetic compound was obtained by extraction of picric acid with ether from a solution of the picrate in 5 per cent sulfuric acid, removal of the sulfate quantitatively with barium hydroxide, evaporation of the solution under reduced pressure to a small volume, and addition of alcohol to a concentration of 80 per cent. The free compound crystallized in small clustered

¹ All melting points are corrected in this and the following two papers.

needles identical in appearance with those of natural octopine. The synthetic material melted with decomposition at 265°, and a mixture with a sample of natural octopine gave no depression.

$C_9H_{13}N_3O_4$. Calculated, N 22.76; found, N 22.59

Synthetic octopine gave a positive Liebermann nitrosamine reaction for secondary amines. It was neutral or very faintly acid in aqueous solution. The specific rotation in water was +19.6°, which agrees well with that of natural octopine.

A picrolonate was formed by the addition of the calculated amount of picrolonic acid to a warm solution of the compound. It crystallized in small, clustered needles which, after recrystallization, melted at 236°. A mixture with the picrolonate of natural octopine showed no depression.

Determination of Titration Curve and Apparent Dissociation Constants of Octopine—Samples of octopine which had been recrystallized four times and which gave satisfactory elementary analyses were used in the determination of the titration curve. The samples were dried to constant weight in a vacuum desiccator.

The titration curve was determined by the use of a glass electrode with improved electron tube potentiometer designed by Stadie, O'Brien, and Laug (13). pH was calculated according to the following equation,

$$E = \frac{RT}{NF} \text{pH} - e \quad (1)$$

in which E is the observed potential in volts, e is the cell constant, and the expression RT/NF is made up of the usual constants and has the value 0.059 at 25°. The cell constant was calculated by the use of Equation 1 with the values of the E.M.F. of several phosphate and borate buffers.

Separate 10 cc. samples of octopine in 0.1 M solution were used for the determination of the curve in the acid and alkaline regions, the titrations being carried out by the use of a microburette graduated in 0.01 cc. with 0.5 N HCl and NaOH, respectively. These data are recorded in Tables I to III and in Fig. 1 which also shows the curve of arginine (from Birch and Harris (3)) for comparison. Both curves have been corrected for the buffering effect of water.

The following equations were used in calculating values for the pK' of the various groups:

$$pK' = pH + \log \frac{[Cl^-] - (H^+)/\gamma}{[C] - ([Cl^-] - (H^+)/\gamma)} \quad (2)$$

$$pK' = pH - \log \frac{[Na^+] - (OH^-)/\gamma}{[C] - ([Na^+] - (OH^-)/\gamma)} \quad (3)$$

in which $[C]$ is the total amino acid concentration, $[Na^+]$ and $[Cl^-]$ are the concentrations of sodium and chloride, in moles per liter, and (H^+) and (OH^-) are the activities of the hydrogen and hydroxyl ions. These equations make allowance for the buffering effect of water. Each value for the activity coefficient

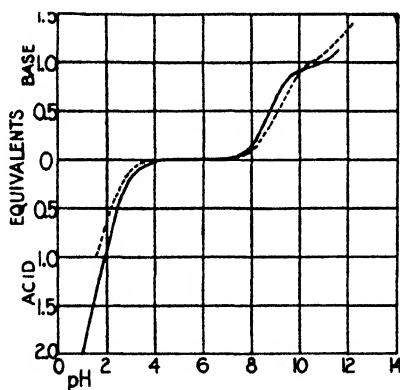


FIG. 1. Titration curves of octopine and arginine. The solid line represents octopine; the broken line, arginine (from Birch and Harris (3)).

(γ) , used in solving the above equations, was calculated on the basis of the total concentration of sodium (or chloride) present at the reading in question, and was assumed to be the same as for an equal concentration of NaOH or HCl in water alone. These values of γ for NaOH and HCl were taken from the papers of Harned (5) and Noyes and MacInnes (12), respectively, and are recorded in Tables I to III.

It may be seen from the curve for octopine (Fig. 1) that there is no plateau between the two groups represented by the acid portion of the curve (the carboxyl groups according to the zwitter

ion hypothesis). This indicates that the carboxyls are of the same or nearly equal strengths and necessitates a consideration of both possibilities in the calculation of the apparent dissociation constants of the groups. First, assuming that they were of the *same* strength, the pK' was calculated from the data for the acid region of the curve. This pK' was not sufficiently constant and showed a systematic variation. This was considered to be an indication that the groups were probably not of the same strength. Therefore, we next made the other possible assumption; *i.e.*, that

TABLE I
Calculation of pK'_{A_2} of Octopine

pH	Volume of HCl	[Cl ⁻] (total)	γ	(H ⁺)/ γ	$\frac{[Cl^-]}{(H^+)/\gamma}$	[C]	pK'_{A_2}
	cc.						
6.25						0.1000	
3.74	0.095	0.0047	0.97	0.0002	0.0045	0.0991	2.41
3.44	0.170	0.0083	0.94	0.0004	0.0079	0.0985	2.38
3.36	0.204	0.0097	0.93	0.0005	0.0092	0.0979	2.38
3.25	0.258	0.0125	0.93	0.0006	0.0119	0.0975	2.39
3.17	0.303	0.0147	0.92	0.0007	0.0140	0.0970	2.39
3.08	0.358	0.0172	0.91	0.0009	0.0163	0.0965	2.39
3.05	0.437	0.0209	0.90	0.0010	0.0199	0.0960	2.47
2.87	0.535	0.0252	0.89	0.0015	0.0237	0.0952	2.39
2.77	0.634	0.0297	0.88	0.0019	0.0276	0.0943	2.39
2.69	0.732	0.0339	0.88	0.0023	0.0316	0.0934	2.40
2.55	0.924	0.0420	0.87	0.0032	0.0388	0.0916	2.41
2.44	1.150						2.40 (average)
2.27	1.428						
2.10	1.794						

the carboxyls were of unequal but closely similar strengths. The dissociation constants of these groups were then calculated by a method similar to that of Hastings and Van Slyke (6). By this method, pK'_{A_2} was calculated from the data for the extreme upper portion of the acid range of the titration curve, *assuming* that this part was due entirely to the second carboxyl and was unaffected by the first. This assumption is more valid when the difference between the strengths of the groups is great. Therefore, the accuracy of the apparent dissociation constants calculated by this method depends both upon the validity of the assumption

just made and the accuracy of the pH determinations. The data for the calculation of pK'_A , are recorded in Table I. This value of pK'_A , (2.40) was then used for the calculation of the amounts of HCl used in repressing the ionization of the *second* carboxyl of octopine in the region of the curve where the two acidic groups *overlap* in their effect. With the values of pH and $[C]$ given in columns (a) and (g) of Table II, these quan-

TABLE II
Calculation of pK'_A , of Octopine

pH (a)	Volume of HCl (b)	[Cl ⁻] (total) (c)	γ (d)	(H ⁺)/ γ (e)
<i>cc.</i>				
1.97	2.119	0.0869	0.84	0.0127
1.92	2.263	0.0918	0.83	0.0146
1.82	2.568	0.1015	0.82	0.0202
1.72	2.884	0.1114	0.82	0.0233
1.63	3.203	0.1209	0.82	0.0286
1.56	3.515	0.1291	0.82	0.0337
1.45	4.037	0.1432	0.81	0.0438
1.41	4.226	0.1479	0.81	0.0481
[Cl ⁻] - (H ⁺)/ γ (f)	[C] (g)	([Cl ⁻] - (H ⁺)/ γ) ₁ calculated (h)	([Cl ⁻] - (H ⁺)/ γ) ₂ = (f) - (h) (i)	pK'_A (j)
0.0742	0.0824	0.0601	0.0141	1.28
0.0772	0.0816	0.0614	0.0158	1.30
0.0813	0.0796	0.0636	0.0177	1.26
0.0881	0.0776	0.0643	0.0238	1.37
0.0923	0.0757	0.0643	0.0280	1.40
0.0954	0.0739	0.0645	0.0309	1.41
0.0994	0.0712	0.0642	0.0352	1.44
0.0998	0.0703	0.0639	0.0359	1.42
				1.36 (average)

ties of HCl for the second group were calculated from Equation 2, being equal to the expression $([Cl^-] - (H^+)/\gamma)_2$. They are recorded in column (h) of Table II. These values were then subtracted from the corresponding quantities of $([Cl^-] - (H^+)/\gamma)$ determined from the *total* HCl added to the solution at a particular pH and recorded in column (f). The difference is $([Cl^-] - (H^+)/\gamma)_1$ (column (i)) which corresponds to the HCl

for the repression of the ionization of the *first* carboxyl at a particular pH. These values with the corresponding ones for $[C]$ and pH were used in calculating pK'_{A_1} (column (j)) with Equation 2.

Since this method of calculating the apparent dissociation constants of the two carboxyls involved an assumption that the extreme upper portion of the acid curve was due solely to the second group and was unaffected by the first, we have attempted to test the accuracy of the calculated constants. When two theoretical curves were drawn by the use of these constants and then combined, the combined curve agreed well with the experi-

TABLE III
Calculation of pK'_{B_1} of Octopine

pH	Volume of 0.502 N NaOH	[Na ⁺] (total)	γ	(OH ⁻)/ γ	$\frac{[Na^+] - (OH^-)}{\gamma}$	[C]	pK'_{B_1}
6.35	0.000					0.100	
7.36	0.076	0.0038	0.95	2.48×10^{-7}	0.0038	0.0993	8.76
7.70	0.153	0.0076	0.94	5.36×10^{-7}	0.0076	0.0985	8.78
7.86	0.223	0.0109	0.92	7.92×10^{-7}	0.0109	0.0978	8.76
8.01	0.305	0.0148	0.90	1.15×10^{-6}	0.0148	0.0970	8.75
8.25	0.478	0.0229	0.88	2.05×10^{-6}	0.0229	0.0955	8.75
8.59	0.824	0.0382	0.85	4.60×10^{-6}	0.0382	0.0924	8.74
8.92	1.178	0.0531	0.82	1.01×10^{-5}	0.0531	0.0895	8.76
9.06	1.315	0.0583	0.81	1.43×10^{-5}	0.0583	0.0884	8.77
9.27	1.498	0.0655	0.80	2.02×10^{-5}	0.0655	0.0871	8.79
9.60	1.620	0.0699	0.80	5.01×10^{-5}	0.0698	0.0860	8.96
9.83	1.798	0.0766	0.79	8.59×10^{-5}	0.0765	0.0848	8.86

mental acid curve. This indicates that, within the limits of the errors of the determinations, the constants seem to be reasonably accurate.

The imino group of octopine has a pK' of 8.76 as calculated from Equation 3 and recorded in Table III. This group is more weakly basic than the α -amino group of arginine (pK' 9.09 according to Birch and Harris (3)). Because of the great difficulty involved in the precise measurement of pH in the very alkaline region, the pK' for the guanidine group of octopine was not determined accurately. However, it appears to be somewhat less than 13 on the basis of the measurements attempted in that region.

We wish to thank Dr. W. C. Stadie for his advice and assistance in determining the titration curve of octopine.

SUMMARY

A synthesis of the natural isomer of octopine has been described.

The titration curve of octopine has been determined and the apparent dissociation constants calculated. The pK values for the two carboxyls are 1.36 and 2.40; those for the two basic groups are 8.76 and somewhat less than 13.

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STUDIES ON OCTOPINE

II. THE NITROGENOUS EXTRACTIVES OF SQUID AND OCTOPUS MUSCLE*

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(Received for publication, November 5, 1938)

Creatine in vertebrates (8, 7) and arginine in invertebrates (19, 15) have been shown to be involved in muscle metabolism. However, besides arginine other guanidine compounds have been isolated from the muscle of certain invertebrates. Guanidine and agmatine were found in extracts of the sponge, *Geodia gygas* (2). Ackermann (1) has obtained from the starfish asterubine, which he has identified as dimethylguanidotaurine. Kutscher, Ackermann, and collaborators (14, 16, 17) have isolated from the muscle of *Arca noæ* arcaine, which was shown to be tetramethylene diguanidine. Morizawa (21), working with *Octopus octopodia*, obtained no arginine but guanidine and a base which he called octopine. Henze (9) may have isolated the latter compound previously from octopus.

Octopine was isolated from scallop muscle in 1934 by Moore and Wilson (20). The structure was determined by them and has been confirmed by syntheses made by Akasi (4) and ourselves (12), working independently. Mayeda (18) has recently reported the isolation of octopine from the Japanese scallop, *Pecten yessensis*. Most recently, Ackermann and Mohr (3) have obtained arginine, octopine, and agmatine from *Eledone moschata*.

In the course of the present study, the extractives of the muscle of the squid, *Loligo pealii*, and of the octopus, *Octopus vulgaris*, have been investigated. We were chiefly interested in the guanidine compounds in these extractives, but other fractions of the

* A preliminary report of this work was presented before the American Society of Biological Chemists at Baltimore, April 2, 1938 (11).

octopus muscle extract were studied for comparison with the results of investigations on the closely related species, *Octopus octopodia*, reported by Morizawa (21).

EXPERIMENTAL

Isolation of Arginine and Octopine from an Extract of Squid Muscle—The squid, *Loligo pealii*, had been preserved on ice for several days after the date of capture. A 50 gm. sample of the hashed mantle and tentacle of the squid was extracted repeatedly with 10 per cent trichloroacetic acid. After neutralization with sodium hydroxide, the solution was analyzed for guanidine bases by the Sakaguchi method as employed by Moore and Wilson (20) and for arginine by the arginase-urease procedure of Hunter and Dauphinee (10). The analyses indicated 0.37 gm. of arginine per 100 gm. of muscle and 0.45 gm. of guanidine compounds by the Sakaguchi method calculated as arginine. The difference, 0.08 gm., indicated that octopine or some similar compound was present in addition to arginine, since octopine gives a color with the Sakaguchi reagents, but cannot be hydrolyzed and determined by arginase extracts prepared from hog liver.

For the purpose of isolation, a 2500 gm. portion of the ground mantle and tentacle of the squid was extracted with warm water several times, and the combined extracts were freed of protein and glycogen with alcohol, and then fractioned as described by Moore and Wilson (20) for the isolation of arginine and octopine with the exception of the omission of the mercury precipitation. 16.1 gm. of arginine flavianate (5.7 gm. of arginine) and 1.5 gm. of octopine picrate (0.78 gm. of octopine) were obtained from the arginine fraction.

The arginine flavianate was converted to the monohydrochloride by the method of Cox (6). The product melted at 220° and a mixture with a known sample showed no depression. 98 per cent of the expected amount of ammonia was obtained by the arginase-urease method.

$C_6H_{14}N_4O_2 \cdot HCl$.	Calculated.	N 26.59,	NH_4-N 6.65
	Found.	" 26.37,	" 6.69

The octopine picrate melted with decomposition at 226° and a mixture with a known sample gave no depression. The picrate

did not contain water of crystallization and no amino nitrogen was found by the Van Slyke method.

$C_8H_{11}N_4O_4 \cdot C_6H_5N_3O_7$. Calculated. N 20.63, picric acid 48.2
Found. " 20.35, " " 48.1

Free octopine was obtained by the usual method. The material crystallized in small clustered needles identical in appearance with those of octopine. It melted with decomposition at 264° , and a mixture with a known sample showed no depression. The compound contained no water of crystallization.

$C_8H_{11}N_4O_4$. Calculated, N 22.76; found, N 22.51

The copper nitrate salt was prepared according to the method of Akasi (4). It crystallized in the form of pale blue needles containing 2 molecules of water. After the removal of the water of crystallization, the compound was analyzed for copper.

$C_8H_{11}N_4O_4 \cdot Cu(NO_3)_2$. Calculated, Cu 14.66; found, Cu 14.52

A hitherto undescribed copper compound of octopine was prepared as follows: An excess of an aqueous suspension of freshly precipitated copper hydroxide was added to a small amount of a concentrated solution of octopine. The mixture was heated for 10 minutes and filtered while hot. The clear deep blue solution was evaporated to a thin syrup in a vacuum desiccator. On long standing in the cold, the copper salt of octopine crystallized in the form of small, light blue needles. The recrystallized material melted with decomposition at $223-227^\circ$. It was moderately soluble in water. After the removal of the 2 moles of water with which the compound crystallizes, it gave the following analyses.

$(C_8H_{11}N_4O_4)_2Cu$. Calculated. Cu 11.48, N 20.23, octopine 89.05
Found. " 11.29, " 19.93, " 88.41

Octopine was determined by the Sakaguchi method after removal of the copper.

A nickel compound of octopine was prepared in a similar manner. The compound did not melt below 290° and contained 87.1 per cent octopine as determined by the Sakaguchi method.

A reineckate of octopine was obtained by adding a slight excess of a cold saturated solution of Reinecke salt to 0.3 gm. of octopine dissolved in a small amount of 0.1 N HCl. The salt crystallized

in the form of pink plates which tended to form clusters. It was filtered on a microsuction funnel and washed with small portions of ice-cold 0.1 N HCl. A yield of 0.22 gm. was obtained. The reineckate of octopine is not precipitated in neutral solution and is much more soluble than that of arginine. The salt was analyzed for octopine by the Sakaguchi method after removal of the Reinecke acid by extraction from an acid solution with a mixture of amyl alcohol and ether. An octopine standard solution was used for comparison.

$C_9H_{18}N_4O_4 \cdot H(Cr(SCN)_4(NH_2)_2)$. Calculated. Octopine 43.6
Found. " 43.1

TABLE I
Nitrogen Partition of Squid Muscle Extract

Fraction	N per 100 gm. muscle			Total N in fraction to total extractive N
	"Arginine" N by Sakaguchi method	Amino N	Total N	
	gm.	gm.	gm.	per cent
Total extract.	0.138	0.236	0.613	100
Purine.		0.001	0.031	5.1
Histidine.		0.005	0.019	3.1
Arginine filtrate.		0.191	0.419	68.4
" (total)	0.130	0.030	0.125	20.4
" phosphotungstate precipitate.	0.117	0.025	0.113	
Flavinate filtrate (octopine)	0.014	0.000	0.011	

Nitrogen Partition of Squid Muscle Extract—250 gm. of mantle and tentacle from the squid were ground and extracted five times with 300 cc. portions of water warmed to 75°. Protein and glyco-gen were removed from the solution by the addition of alcohol. After repeated evaporation of the filtrate for the removal of alcohol, the solution was freed of ammonia by boiling under reduced pressure after being made slightly alkaline with barium hydroxide. Then the solution was analyzed for total nitrogen by the Kjeldahl method, for amino nitrogen by the Van Slyke procedure, and for guanidine bases by the Sakaguchi method as used by Moore and Wilson (20). The results of these analyses are given in the first line of figures of Table I. The solution was then fractioned by the

Kossel and Kutscher procedure with silver sulfate and barium hydroxide. Four fractions were obtained: purine (pH 3), histidine (pH 7 to 7.2), arginine (pH 10), and arginine filtrate (the fraction not precipitated by silver and baryta). After removal of silver from each fraction with hydrogen sulfide, the solutions were analyzed for total and amino nitrogen. A Sakaguchi analysis was carried out on the arginine fraction. All results are recorded in Table I. The arginine fraction was precipitated with phosphotungstic acid. After centrifuging and washing, the precipitate was decomposed with barium hydroxide in the usual manner, and barium was removed with sulfuric acid. Kjeldahl, Van Slyke amino nitrogen, and Sakaguchi analyses were made on the phosphotungstic acid fraction. Then the arginine in the solution was removed with flavianic acid. After extraction of the excess flavianic acid from the filtrate, the solution was analyzed as before. The results are recorded in Table I. In the last column are listed the percentages of total extractive nitrogen as divided among the various fractions.

It can be seen that the arginine filtrate fraction contains the greatest percentage of the total extractive nitrogen, while the arginine fraction contains the next largest amount. The analyses indicate that the arginine fraction consists of one or more compounds in addition to arginine. As described above, octopine has been isolated from this fraction and its presence may explain the results.

Nitrogenous Extractives of Octopus Muscle—The octopus muscle used in this work was from the arms of several animals sent to us from Bermuda by Dr. F. G. Wheeler. The muscle had been preserved in 95 per cent alcohol, but had not been weighed while fresh. The preserved muscle weighed 2575 gm., which was undoubtedly less than the weight of the fresh tissue. The alcohol preservative was concentrated by vacuum distillation and the residue was added to the main extract.

The isolation procedure is not given in great detail, since the methods have been described before. The hashed muscle was extracted repeatedly with warm water, and, after concentration, protein was removed from the solution with tannic acid. Excess tannic acid was removed in the usual way, and the solution was treated with phosphotungstic acid. The filtrate from the phos-

phosphotungstic acid. On standing in the ice box overnight, taurine precipitated in the form of thick needles. The recrystallized compound contained sulfur and gave the following analyses.

$C_2H_7NO_2S$. Calculated. N 25.63, NH_2-N 25.63
 Found. " 25.41, " 25.35

The acidified taurine filtrate was extracted repeatedly with ether. From the combined ether extracts a small amount of a zinc salt was prepared. It gave a positive Uffelman lactic acid reaction and was optically inactive in aqueous solution.

The solution which had been extracted with ether was acidified with hydrochloric acid and boiled for 3 hours on a water bath. The solution gave a negative Jaffe reaction for creatinine. This experiment contrasts with that of Morizawa who reported the presence of creatinine in a muscle extract of *Octopus octopodia* treated in the manner just described.

The phosphotungstic acid precipitate was decomposed with barium hydroxide in the usual manner. After the removal of excess barium, the solution was fractioned with silver nitrate and barium hydroxide according to the Kossel and Kutscher procedure. Four fractions were obtained: purine (pH 3), histidine (pH 7 to 7.2), arginine (pH 10), and lysine (the fraction not precipitated by silver and baryta). The lysine fraction was freed of silver and barium and was reprecipitated with phosphotungstic acid.

Purine Fraction

Separation of the constituents of this fraction was accomplished by the method of Krüger and Schittenhelm (13). A picrate melting with decomposition at 282° was obtained and identified as adenine picrate.

$C_8H_8N_6 \cdot C_6H_3N_3O_7$. Calculated, N 30.77; found, N 30.56

A sample of the compound gave a positive Kossel test after removal of picric acid with ether.

Hypoxanthine was obtained as the nitrate which formed characteristic crystals with curved edges, the so called whetstone crystals. Some of the material was converted into the picrate which decomposed at 257° after recrystallization.

$C_8H_8N_6O \cdot C_6H_3N_3O_7$. Calculated, N 26.85; found, N 26.67

Arginine Fraction

The details of the isolation of the constituents of this fraction are presented, since this was the fraction in which we were most interested. The silver precipitate was suspended in water, made acid with sulfuric acid, and decomposed with hydrogen sulfide. The silver sulfide was centrifuged and washed several times with equal volumes of water. The filtrate and washings were made faintly acid to Congo red with sulfuric acid and were treated with a concentrated solution of flavianic acid until precipitation was complete. The flavianate which separated on standing overnight had the typical plate-like form of arginine flavianate. The arginine flavianate was converted to the monohydrochloride by the method of Cox (6). This salt melted at 222° with decomposition and a mixture with a known sample showed no depression. 98.5 per cent of the expected amount of ammonia was obtained by the arginase-urease method.

$C_6H_{14}N_4O_2 \cdot HCl$.	Calculated.	N 26.59,	NH_2-N 6.65
	Found.	" 26.47,	" 6.59

The flavianate filtrate was freed of flavianic acid by extraction with butyl alcohol-ether mixture after the addition of sulfuric acid to a concentration of 5 per cent. After removal of sulfate with barium hydroxide, the solution was evaporated *in vacuo* to a thin syrup, and alcohol was added to a concentration of 80 per cent. On standing overnight in the ice box, a white crystalline material separated out in the form of small clustered needles. After several recrystallizations, the compound melted at 264° , and a mixture with a known sample of octopine gave no depression. The specific rotation of the compound in aqueous solution was $+20.1^{\circ}$. The material was neutral to litmus and gave no free amino nitrogen when analyzed by the Van Slyke method.

$C_8H_{12}N_4O_4$.	Calculated,	N 22.76;	found, N 22.41
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With picric acid the compound formed yellow anhydrous needles melting with decomposition at 227° . The melting point of a mixture with octopine picrate was not depressed.

$C_8H_{12}N_4O_4 \cdot C_6H_3N_3O_7$.	Calculated.	N 20.63,	picric acid 48.2
	Found.	" 20.29,	" " 48.3

The compound gave a theoretical analysis by the Sakaguchi method as used by Moore and Wilson (20). The color given

with the Sakaguchi reagents was the typical purple color given by octopine. Hog liver arginase preparations did not produce urea from the compound, and a positive Liebermann test for secondary amines was obtained.

The picrolonate of the compound crystallized as burrs of small yellow needles. It melted with decomposition at 237° , and a mixture with a known sample of octopine picrolonate gave no depression. All these data demonstrate that the compound was octopine.

The filtrate from the precipitation of octopine with alcohol was then investigated. The remaining traces of octopine were precipitated with mercuric sulfate and additional alcohol. The filtrate was freed of alcohol through evaporation *in vacuo*, of mercury by hydrogen sulfide, and of sulfate with barium hydroxide. The solution was analyzed by the Sakaguchi, the Van Slyke amino nitrogen, and the arginase methods. The Sakaguchi analyses indicated about 0.2 gm. of guanidine compounds, calculated as arginine, when an arginine standard was used. However, the arginase determination indicated that arginine was almost completely absent. The Van Slyke amino nitrogen determination, calculated as arginine for comparison with the Sakaguchi analysis, was about 0.13 gm. These analyses seemed to indicate the presence of one or more non-arginine compounds giving a positive Sakaguchi reaction. Some material containing free amino nitrogen was also present. Since Ackermann and Mohr (3) had obtained agmatine from the arginine fraction of an extract of *Eledone moschata*, we examined our extract for this compound. For this purpose we employed a procedure involving the formation of benzylidene agmatine. It was produced by the general method of Bergmann (5) for amino acids. The compound was then extracted with ether, decomposed by acid hydrolysis, and agmatine was obtained finally as the picrate. The details of our procedure are given below.

The solution (15 cc. volume) was treated with sodium hydroxide until a concentration of 5 per cent was reached. After cooling to 0° in an ice bath, 1 cc. of benzaldehyde was added and the mixture was stirred mechanically for 12 hours. The alkaline solution was then extracted four times with 100 cc. portions of ether. The ether extract was acidified with dilute sulfuric acid, the ether was

evaporated under reduced pressure, and the solution was heated gently on the water bath for 1 hour in order to hydrolyze the benzylidene agmatine. After cooling, the solution was treated with phosphotungstic acid in 5 per cent sulfuric acid for the precipitation of agmatine. The phosphotungstate was decomposed with barium hydroxide in the usual manner. An excess of a saturated solution of picric acid was added to the barium-free solution, and on standing in the ice box overnight, an orange-colored picrate crystallized in the form of leaves. After recrystallization, the picrate melted with decomposition at 236° and a mixture with a known sample of agmatine picrate showed no depression.

$C_8H_{14}N_4 \cdot (C_6H_3N_3O_7)_2$. Calculated. N 23.81, NH_2-N 2.38, picric acid 77.88
 Found. " 23.59, " 2.32, " " 77.31

Lysine Fraction

From the lysine fraction betaine was isolated as the picrate. After recrystallization, the picrate melted at 182° and gave the following analyses.

$C_8H_{11}NO_2 \cdot C_6H_3N_3O_7$. Calculated. N 16.19, picric acid 33.83
 Found. " 16.08, " " 33.71

The picrate was converted to the hydrochloride which was insoluble in absolute alcohol and melted at 182° after recrystallization.

$C_8H_{11}NO_2 \cdot HCl$. Calculated, N 9.12; found, N 9.03

SUMMARY

1. Both arginine and octopine have been isolated from aqueous extracts of the mantle and tentacle muscle of the squid, *Loligo pealii*. Three new salts of octopine are described.

2. The nitrogen partition of the extractives of squid muscle has been determined.

3. Taurine, adenine, hypoxanthine, betaine, arginine, octopine, and agmatine were obtained from an extract of the tentacle muscle of the octopus, *Octopus vulgaris*. The isolation of these compounds is in agreement with the work of Morizawa and of Ackermann and Mohr on related animals.

4. A new method for the isolation of agmatine is reported.

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STUDIES ON OCTOPINE

III. THE PRECURSOR OF OCTOPINE IN AUTOLYZING SCALLOP MUSCLE*

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Moore and Wilson (3) found that muscle dissected from live scallops contained large amounts of arginine and only small amounts of octopine. On the other hand, they found that scallop muscle purchased from the market contained large amounts of octopine and little or no arginine. This apparent reciprocal relationship between the concentrations of arginine and octopine suggests that arginine may be the precursor of octopine. The results of a further investigation of the problem are presented in this paper.

It was found that arginine, which, in fresh muscle dissected from live scallops, constituted about 84 per cent of the total guanidine bases analyzable by the Sakaguchi method, decreased to about 10 per cent of the total during autolysis of the muscle slices for 3 days at 0°. During this period the amount of octopine increased reciprocally. These results were obtained by analyses and were confirmed by isolations.

EXPERIMENTAL

The preparation of the material was carried out in a cold room as rapidly as possible. The adductor muscles of live scallops, *Pecten magellanicus*, were dissected out, and the tissue was divided into four portions of about 300 gm. each. Two portions of the tissue were sliced into pieces 2 or 3 mm. in thickness. Toluene

* A preliminary report of this work was presented before the American Society of Biological Chemists at Baltimore, April 2, 1938 (2).

was added to one portion and omitted from the other. The remaining two were ground in a meat chopper and placed in separate flasks, toluene being added to one and omitted from the other. Approximately 50 gm. samples of the tissue from each autolyzing flask were removed immediately, weighed, ground, and extracted six times with 100 cc. portions of 10 per cent trichloroacetic acid. The extracts were then neutralized with sodium hydroxide, made up to suitable volumes, and analyzed by the arginase-urease method of Hunter and Dauphinee (1) and by the

TABLE I
Formation of Octopine in Autolyzing Scallop Muscle

The results (except those in Column 4) are expressed as gm. of arginine per 100 gm. of muscle.

Duration of autolysis	Sakaguchi determination	Arginase determination	Arginase to Sakaguchi determination	Silver-baryta fraction		
				Sakaguchi determination	Arginase determination of flavianate ppt.	Octopine isolated
(1)	(2)	(3)	(4)	(5)	(6)	(7)
	gm.	gm.	per cent	gm.	gm.	gm.
Original slices.....	0.98	0.81	84	0.85	0.69	0.14
Slices, 18 hrs.	1.04	0.64	62	0.90	0.56	
“ 44 “	1.03	0.42	41	0.88	0.36	
“ 65 “	1.21	0.12	10	1.00	0.11	0.71
“ + toluene, 70 hrs.....	1.11	0.09	8	0.91	0.09	0.67
Hashed tissue + toluene, 73 hrs.....	1.05	0.64	61	0.90	0.56	
Hashed tissue + toluene, 22 days	1.05	0.41	39	0.89	0.36	0.47

Sakaguchi method as used in our laboratory (3). Both arginine and octopine give a color with the Sakaguchi reagents which can be quantitatively compared with the color produced by an arginine standard when the color filter is used. On the other hand, the arginase-urease method indicates the amount of arginine alone, since the hog liver arginase preparation does not produce urea from octopine. The difference between the two analyses was assumed to be octopine. The results of these analyses of the original muscle before autolysis can be seen in the first line of

figures in Columns 2 and 3 of Table I. The results are expressed in gm. of arginine per 100 gm. of muscle. The percentage of the total guanidine (Sakaguchi method) which is arginine is given in Column 4. It can be seen from Table I that before autolysis the amount of total guanidine as determined by the Sakaguchi method was equivalent to about 1 gm. of arginine per 100 gm. of muscle. About 84 per cent was true arginine. The remainder, or 16 per cent of the total, was octopine, if we assume that octopine was the only other compound determined by the Sakaguchi method.

After these initial analyses, the various portions of tissue were permitted to autolyze in the cold room, and samples were removed at frequent intervals for analysis by the methods just described. A few of the representative determinations are recorded in the appropriate columns of Table I. The autolyzing flask from which the sample was removed, and the duration of the autolysis at the time of the removal of the sample, are given in Column 1. Thus in the tissue slices the total guanidine determined by the Sakaguchi method and recorded in Column 2 did not decrease during autolysis. Indeed, it apparently increased, which is to be expected if octopine were formed from arginine, since, with the Sakaguchi reagents, octopine produces a 20 per cent greater result than an equimolar quantity of arginine. On the other hand, the proportion of arginine in the tissue slices decreased rapidly, as indicated by the arginase determinations listed in Column 3. The proportion of arginine fell from an initial value of about 84 per cent of the guanidine by the Sakaguchi method to 10 per cent in 65 hours of autolysis of the slices. Reciprocally, the amount of octopine increased during the same period as shown by the increasing difference between the Sakaguchi and arginase-urease determinations, assuming that this difference is largely due to octopine. The autolysis of the tissue slices to which toluene had been added resulted in a similar formation of octopine from arginine, indicating that toluene does not affect the reaction. The rate of octopine formation was slower in the hashed tissue than in the tissue slices.

As a check on the validity of the conclusions which were drawn from the results of the arginase-urease and Sakaguchi determinations, additional analyses and isolations of octopine were carried out on the extracts as follows: The main portions of the extracts (after removal of samples for the preliminary analyses) were

treated with silver nitrate and barium hydroxide (pH 10) in order to precipitate both arginine and octopine. The precipitate was removed by centrifugation and was washed several times with 75 cc. portions of saturated barium hydroxide solution. The precipitate was suspended in water, made acid with sulfuric acid, and decomposed with hydrogen sulfide. The filtrate from the silver sulfide was analyzed by the Sakaguchi method. The value for this analysis, again expressed as gm. of arginine per 100 gm. of muscle and recorded in Column 5, gives an indication of the amount of loss sustained in the precipitation with silver-baryta. The solution was made just faintly acid to Congo red with sulfuric acid and was treated at the boiling point with a saturated aqueous solution of flavianic acid for the precipitation of arginine. After standing overnight in an ice box, the flavianate precipitate was filtered off, washed several times with small portions of dilute flavianic acid, dried, and weighed. Samples of the flavianate precipitate were analyzed by the arginase method after the flavianic acid was extracted with butyl alcohol and ether. The figures for arginine thus determined are shown in Column 6. These values were checked by calculation of arginine from the weight of the flavianate and by analysis of the flavianate samples by the Van Slyke amino nitrogen method. Suitable agreement was obtained, but the results are not recorded. All results were corrected for the solubility of arginine flavianate. Finally octopine was isolated as the picrate from the flavianate filtrates after extraction of the flavianic acid with butyl alcohol and ether. The amount of octopine is given in Column 7 after correction for solubility of octopine picrate. The actual figures are calculated as gm. of arginine in order to permit comparison with the other figures. The results of these additional analyses and isolations confirm the original conclusions drawn from the Sakaguchi and arginase-urease determinations. Thus in the tissue slices before autolysis a large amount of arginine was obtained as the flavianate, but only a small amount of octopine was isolated as the picrate. After autolysis of the slices for 65 hours, the quantity of arginine had decreased to a very small amount, while the proportion of octopine had increased reciprocally, as shown by the isolation of a relatively large amount of this compound as the picrate. The octopine picrate samples were identified by comparison of melting

points with a known sample, by micro-Kjeldahl analyses, and by isolation of the free compound after removal of picric acid.

SUMMARY

1. Evidence has been presented that arginine is the precursor of octopine in autolyzing scallop muscle.

2. The rate of formation of octopine is more rapid in sliced than in hashed tissue.

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THE HEAT OF OXYGENATION OF HEMOGLOBIN

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Measurements of the heat of the reaction of hemoglobin with oxygen have been carried out by a number of investigators. The most comprehensive work appears to be that of Roughton and his collaborators (1, 2) who have studied the problem both by a direct calorimetric method and by application of the van't Hoff equation to observations on the effect of temperature on the dissociation pressure of oxyhemoglobin. Their measurements were carried out at pH 6.8 and 9.5. At both pH values the heats obtained by the two methods agree to within the errors of the experiments. The directly measured heat of reaction of 1 mole of oxygen with a large amount of hemoglobin is found to be independent of the percentage saturation of the hemoglobin with oxygen. This accords with the fact that the oxygen dissociation curves obtained at a given pH but at different temperatures may all be made to coincide by proper choice of the scale of oxygen pressure for each. The heat liberated per mole of oxygen at pH 6.8 is 9350 calories; that at pH 9.5 about 13,000 calories. The difference is attributed to the heat of the dissociation of hydrogen ion which accompanies oxygenation at pH 6.8 but not at 9.5. The same results were obtained with solutions of purified hemoglobin as with whole blood and laked blood.

In the present paper we shall deal also with the problem of the heat of oxygenation of hemoglobin, but from a somewhat more general point of view and on the basis of additional data. We shall consider in particular how this heat varies with pH over the entire range from pH ~ 3 to pH ~ 11 . From this we shall be led to questions involving the nature and number of the groups whose acid strength is affected by oxygenation. Finally we shall deal with free energy of oxygenation in its relation to pH. The

study will be confined to solutions of crystallized hemoglobin of the horse.

It is known from determinations of molecular weight that each molecule of hemoglobin is capable of combining in all with 4 molecules of oxygen. It follows from this that the most general expression for y , the percentage saturation of hemoglobin with oxygen, in terms of p , the partial pressure of the oxygen, is that given by Adair (3) in terms of four equilibrium constants L .

$$(1) \quad \frac{y}{100} = \frac{L_1 p + 2L_2^2 p^2 + 3L_3^3 p^3 + 4L_4^4 p^4}{4(1 + L_1 p + L_2^2 p^2 + L_3^3 p^3 + L_4^4 p^4)}$$

In general, at any given partial pressure of oxygen, the hemoglobin will be present in five different forms distinguished by the number (0, 1, 2, 3, 4) of molecules of oxygen combined with each molecule of hemoglobin. If we refer to these by Hb, HbO₂ . . . Hb(O₂)₄, we may say that L_1 , L_2^2 , L_3^3 , L_4^4 refer to the equilibria between Hb and HbO₂ . . . Hb(O₂)₄ respectively. Actually it is possible to fit the experimental results very exactly by Equation 1 (4). Since each L is then found to be different from zero it follows that all five forms of hemoglobin are in fact present. Each L is made up of the individual constants describing the equilibria of the four distinct oxygen-combining groups of the hemoglobin molecule, but this does not affect the generality of Equation 1 which holds whether or not the different groups have different constants and whether or not the constant of each group is affected by oxygenation of the other groups. It is known from experiment that, for a given value of p , y varies with pH as well as with temperature. This means that there is an interaction between some at least of the groups which dissociate hydrogen ions and those which combine with oxygen, and shows that we must regard each L as a function of pH as well as of the temperature.

There is a particular feature of the effect of temperature and of pH on the oxygen dissociation curves which leads to a very simple relation between p and the L 's and is of the first importance for our problem. It is shown by the work of Ferry and Green (5) and by that of Roughton and his collaborators (2) that the oxygen dissociation curves obtained at a given temperature but at

different pH values, or at a given pH but different temperatures, may all be made to coincide if in each case the values of p are multiplied by a suitably chosen constant. It follows from this that

$$(2) \quad \left(\frac{\partial \ln L_1}{\partial T} \right)_{\text{pH}} = \dots = \left(\frac{\partial \ln L_4}{\partial T} \right)_{\text{pH}} = \left(\frac{\partial \ln 1/p}{\partial T} \right)_{\text{pH}, y}$$

and

$$(3) \quad \left(\frac{\partial \ln L_1}{\partial \text{pH}} \right)_T = \dots = \left(\frac{\partial \ln L_4}{\partial \text{pH}} \right)_T = \left(\frac{\partial \ln 1/p}{\partial \text{pH}} \right)_{T, y}$$

A formal proof of these almost self-evident relations is the following. Equation 1 shows that y is a zero order homogeneous function of L_1, \dots, L_4 and $1/p$. Consequently

$$(4) \quad \frac{\partial y}{\partial \ln L_1} + \dots + \frac{\partial y}{\partial \ln L_4} + \frac{\partial y}{\partial \ln 1/p} = 0$$

Consider now the effect of changing the temperature by a factor α from T to αT while pH is kept constant. Then we know that y remains unchanged if at the same time we multiply all the values of p by a factor β which depends only on T and α but not on p (or y). Thus

$$(5) \quad y(T, p) = y(\alpha T, \beta p)$$

By differentiating y with respect to α and then letting $\alpha = 1$, we obtain

$$(6) \quad T \left(\frac{\partial y}{\partial T} \right)_p + p \left(\frac{\partial y}{\partial p} \right)_T \left(\frac{\partial \beta}{\partial \alpha} \right)_{\alpha=1} = 0$$

$(\partial \beta / \partial \alpha)_{\alpha=1}$ depends only on the temperature and may be written as $Tf(T)$. Equation 6 may then also be written in the form

$$(7) \quad \left(\frac{\partial y}{\partial \ln L_1} \right)_p \left(\frac{\partial \ln L_1}{\partial T} \right) + \dots + \left(\frac{\partial y}{\partial \ln L_4} \right)_p \left(\frac{\partial \ln L_4}{\partial T} \right) + f(T) \left(\frac{\partial y}{\partial \ln 1/p} \right) = 0$$

Combination of this with Equation 4 gives

$$(8) \quad \left(\frac{\partial y}{\partial \ln L_1} \right) \left(f(T) - \frac{\partial \ln L_1}{\partial T} \right) + \dots + \left(\frac{\partial y}{\partial \ln L_4} \right) \left(f(T) - \frac{\partial \ln L_4}{\partial T} \right) = 0$$

Since this equation holds for all values of p and T , while the expressions in parentheses depend only on T , and since, as may be seen from Equation 1, each of the quantities $\partial y / \partial \ln L_1, \dots \partial y / \partial \ln L_4$ is a function of p of higher degree than the preceding, it follows that each of the expressions in parentheses must vanish and therefore that

$$(8-a) \quad \frac{\partial \ln L_1}{\partial T} = \frac{\partial \ln L_2}{\partial T} = \dots = f(T)$$

At the same time it follows from Equation 6 and the definition of $f(T)$ that

$$(9) \quad f(T) = - \left(\frac{\partial y}{\partial T} \right)_p \left(\frac{\partial \ln p}{\partial y} \right)_T = \left(\frac{\partial \ln p}{\partial T} \right)_y$$

Consequently we have what we set out to prove; namely, Equation 2. Exactly the same procedure, in which pH is substituted for T , leads to Equation 3.

Let us now introduce the symbols Q_1, Q_2, Q_3, Q_4 to refer to the amounts of heat absorbed due to the combination of 1, 2, 3, and 4 moles of oxygen respectively with 1 mole of hemoglobin at constant temperature and pH. Then, since by the van't Hoff equation

$$(10) \quad Q_1 = RT^2 \frac{\partial \ln L_1}{\partial T}, \quad Q_2 = RT^2 \frac{\partial \ln L_2^2}{\partial T}, \quad \text{etc.}$$

it follows from Equation 2, if we change from natural to Briggsian logarithms, that

$$(11) \quad Q_1 = \frac{Q_2}{2} = \frac{Q_3}{3} = \frac{Q_4}{4} = 2.303 RT^2 \left(\frac{\partial \log 1/p}{\partial T} \right)_{y, \text{pH}} = \frac{Q}{4}$$

This means that the heat absorbed is the same for each stage of the process involving the combination of 1 mole of hemoglobin with 4 moles of oxygen and equal to one-quarter of the total heat Q absorbed in the whole process.

Let us next introduce the symbols $B_r, B_1, \dots B_4$ to denote the number of equivalents of base bound at any given temperature

and pH per mole of Hb, HbO₂, . . . Hb(O₂)₄ respectively. We have shown in a previous paper (6) that

$$(12) \quad \frac{\partial \log L_1}{\partial \text{pH}} = B_1 - B_r, \quad \frac{\partial \log L_2}{\partial \text{pH}} = \frac{B_1 - B_r}{2}, \text{ etc.}$$

Consequently it follows from Equation 3 that

$$(13) \quad B_1 - B_r = \dots = \frac{B_4 - B_r}{4} = \left(\frac{\partial \log 1/p}{\partial \text{pH}} \right)_{\text{pH}} = \frac{\Delta B}{4}$$

This shows that the shift in base bound per mole of hemoglobin produced by combination with each successive mole of oxygen is the same and equal to one-quarter of the total shift ΔB produced by complete oxygenation.

We are now in a position to write a general expression for the variation of the heat of oxygenation with pH. Since

$$\frac{\partial}{\partial \text{pH}} \left(\frac{\partial \log 1/p}{\partial T} \right) = \frac{\partial}{\partial T} \left(\frac{\partial \log 1/p}{\partial \text{pH}} \right)$$

it follows from Equations 11 and 13 that

$$(14) \quad \frac{\partial Q}{\partial \text{pH}} = 2.303 RT^2 \left(\frac{\partial \Delta B}{\partial T} \right)_{\text{pH}}$$

We shall make use of this equation, together with experiments to be described presently, in order to determine the variation in the heat of oxygenation from pH ~ 4 to ~ 11 , and in particular to answer the question whether this variation can be accounted for by the heat of hydrogen ion dissociation which is known to be coupled with oxygenation within this range. For this purpose, however, we shall employ Equation 14 in an integral form. Let Q_0 be the heat of oxygenation at some strongly acid pH = pH₀, where the acid dissociation of the protein is unaffected by oxygenation and $\Delta B = 0$, and Q_x the heat at some other pH = pH_x where $\Delta B = \Delta B_x$, then

$$(15) \quad Q_x - Q_0 = 2.303 RT^2 \int_{\text{pH}_0}^{\text{pH}_x} \left(\frac{\partial \Delta B}{\partial T} \right)_{\text{pH}} d\text{pH}$$

If we make use of the identity

$$\left(\frac{\partial \Delta B}{\partial T}\right)_{\text{pH}} = - \left(\frac{\partial \Delta B}{\partial \text{pH}}\right)_T \left(\frac{\partial \text{pH}}{\partial T}\right)_{\Delta B}$$

and integrate by parts, we obtain

$$(16) \quad \frac{Q_s - Q_o}{\Delta B_s} - 2.303 RT^2 \left[\left(\frac{\partial \text{pH}}{\partial T}\right)_{\Delta B} - \frac{1}{\Delta B_s} \int_{\text{pH}_o}^{\text{pH}_s} \Delta B \frac{\partial}{\partial \text{pH}} \left(\frac{\partial \text{pH}}{\partial T}\right)_{\Delta B} d\text{pH} \right]$$

In order to determine the effect of temperature on ΔB , titration curves of oxygenated and reduced hemoglobin of the horse have been made at three temperatures: 7°, 25°, and 38°. Crystalline protein was prepared from red blood cells kindly furnished by the Massachusetts Antitoxin and Vaccine Laboratory by the same procedure as in earlier studies (6, 7). This was dissolved in 0.3 M NaCl to give the stock solutions used for the titrations. These solutions were always kept in the cold and aliquots were withdrawn for titration as the experiments proceeded. The total concentration of protein in each solution was determined by nitrogen analysis. It varied between 74 and 101 gm. per liter. The percentage of inactive hemoglobin was determined at the end of each experiment on the basis of a determination of oxygen capacity with a Van Slyke apparatus, as in earlier studies (6, 7). In one experiment this amounted to 10 per cent.¹ In all the other experiments it was 2 per cent or less. No attempt was made to allow for it in calculating the amount of base bound by oxyhemoglobin, since the corrections involved are less than the experimental error.

Titrated aliquots of solution were divided into two parts, one of which was reduced, the other oxygenated, before injection into the electrode, in accordance with a procedure already described (6, 7). During titration enough water was always added to each aliquot in addition to acid or base so that the total dilution was always the same. This involved an increase of volume to 132 per cent of the initial volume. The pH was measured with a glass electrode. The details of the measurements, including the

¹ The experiment at 7°, represented by squares in Fig. 1.

temperature control and the calibration of the electrode with standard buffers, were the same as in an earlier study (7).

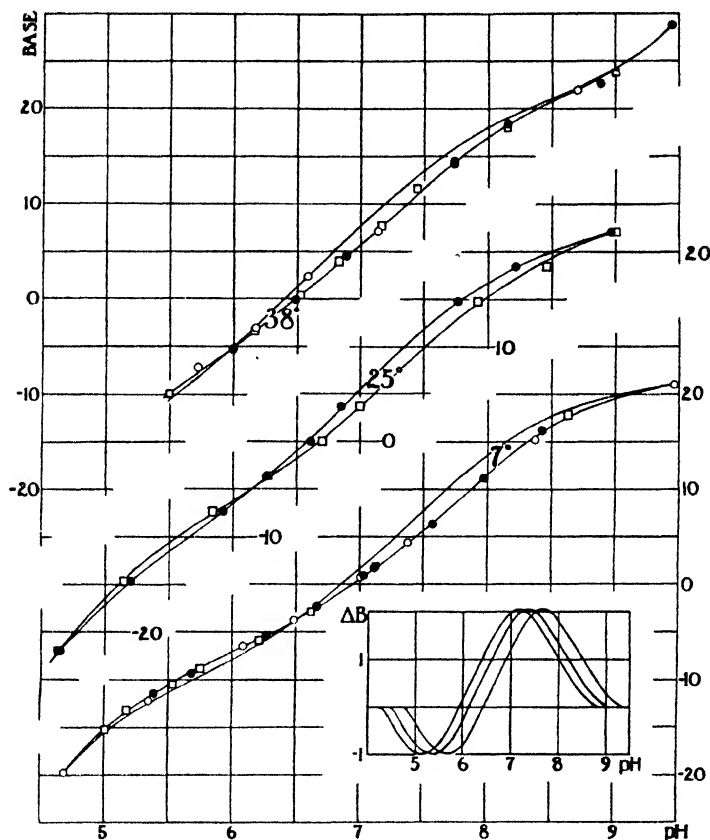


FIG. 1. Titration curves of oxygenated and reduced hemoglobin at different temperatures. The ordinates represent equivalents of base per mole (66,800 gm.) of hemoglobin. For 25° the squares represent reduced hemoglobin, the circles oxygenated hemoglobin. For 7° and 38° all points plotted are for reduced hemoglobin, and different symbols correspond to different experiments.

The results of seven experiments are shown in Fig. 1. The quantity which is directly measured in these experiments is pH and the significant feature of the results is the difference in pH

of a given titrated aliquot in the reduced and oxygenated conditions. In combining the results of different experiments at the same temperature a complication arises due to the fact that there are nearly always minor differences of shape in the titration curves, whether of oxygenated or reduced hemoglobin, obtained with different preparations. For this reason, in the case of the measurements at 7° and at 38°, which involve different stock solutions and preparations, we have constructed a composite titration curve for oxyhemoglobin at each of the two temperatures by drawing in free-hand a curve based on all the observations on oxyhemoglobin at that temperature. In Fig. 1 therefore no points are shown for oxyhemoglobin at 7° and 38°. The only points which are plotted are for reduced hemoglobin. They are located with reference to the composite curve for oxyhemoglobin at each temperature from the observed difference of pH of the oxygenated and reduced forms. The ordinate of each is of course fixed by the amount of acid or base present. This procedure serves to bring together the different sets of measurements at each temperature in a satisfactory way and introduces no appreciable error. The pH shift produced by oxygenation is the only significant factor in the determination of ΔB , for small differences in the shape of the titration curve of oxyhemoglobin have no appreciable effect. The results given for 38° do not extend below pH 5.5, since at this temperature hemoglobin undergoes an irreversible change very rapidly at acid reactions, which renders the data unreliable. The procedure of constructing a composite curve was unnecessary for the data at 25° which were obtained from a single experiment. Only one experiment was made at 25°, because of the extensive earlier work at this temperature. In Fig. 1 the smooth curves for 25° are drawn to give the values of ΔB obtained in the earlier work (6), and show that the present results are in good agreement with it.

Careful measurement of these results shows that the only effect of changing the temperature is to displace by a constant amount the pH values at which given values of ΔB occur. The smooth curves for reduced hemoglobin at 7° and 38° are drawn so that the displacement amounts to +0.30 pH unit for the change from 25° to 7° and to -0.20 unit for the change from 25° to 38°, on the basis of the data for ΔB as a function of pH at 25° given by earlier

work. The ΔB -pH curves used for this purpose are shown in the inset in Fig. 1.

If we revert now to Equation 16, we see, on the basis of these results, that of the two expressions in brackets the first is constant and equal to -0.016 and the second is, therefore, equal to zero. Consequently, at 25° ,

$$\frac{Q_x - Q_o}{\Delta B_x} = 6500 \text{ calories}$$

This value is subject to an uncertainty of at least 10 per cent. The fact that $Q_x - Q_o$ is strictly proportional to ΔB_x shows that the variation in the heat of oxygenation with pH can be accounted for entirely on the basis of the heat of the dissociation of hydrogen ion with which oxygenation is coupled. Whenever $\Delta B_x = 0$, as at strongly basic reactions and at the pH at which the titration curves of oxygenated and reduced hemoglobin cross, $Q_x = Q_o$. The figure 6500 gives the heat of dissociation of the base-binding groups which interact with the oxygen-combining centers of the protein molecule. In a previous paper we have studied the apparent heat of dissociation of hydrogen ion by oxyhemoglobin at 25° between pH 4 and 10. In the middle part of the range this is found to be 6200 calories per equivalent, which is the value characteristic of the imidazole group of histidine. We have interpreted this to mean that the middle portion of the titration curve of oxyhemoglobin is due to the imidazole groups of the thirty-three histidine residues which are known to occur in each molecule of hemoglobin. On the basis of a more detailed analysis we have concluded that in oxyhemoglobin the pK of the weakest imidazole group is certainly less than 7.5 and that of the strongest in the neighborhood of 6. These conclusions accord well with the results of the present study. The figures 6500 and 6200 agree better than might be expected from the experimental errors. This is strong evidence that the base-binding groups which interact with the oxygen-combining groups are all imidazole groups of histidine. This conclusion may be somewhat unexpected in view of the fact that some of these groups are rendered more acid, others more basic, by oxygenation, a fact which follows from the crossing of the titration curves of oxygenated and reduced protein.

The hemoglobin molecule is known to contain four hemes and it is generally believed that these are the four oxygen-combining groups. This of itself suggests a certain degree of symmetry of the hemoglobin molecule, as if it consisted of four quadrants, one for each heme. This suggestion receives support from two facts which have been dealt with above; namely, that the heat absorbed is the same for each stage in the process involving the combination of 1 mole of hemoglobin with 4 moles of oxygen, and that the shift in the amount of base bound by the protein caused by each stage of the process is also the same. At least these facts lead us to believe that each of the oxygen-combining groups is related in the same way to a certain number of histidine units with which there is an interaction. If so, there must be at least two such units for each heme, one of which is rendered more acid, the other more basic, by oxygenation.

Let us now assume that there are in fact just two histidine units which interact with each heme, and that the behavior of each heme and its associated histidines is identical with that of the others. Then it should be possible to fit the ΔB -pH data satisfactorily by a suitable choice of four apparent dissociation constants k' , one for each histidine when the associated heme is in the reduced condition, one for each histidine when the associated heme is in the oxygenated condition. If the data were perfect they should of course determine these four k' values uniquely. The basis for determining the values of k' from the observed values of ΔB is the following. The shift in base bound by a weak acid at any given hydrogen ion activity H in consequence of a shift of its apparent dissociation constant from k'_1 to k'_2 is given by

$$(17) \quad \Delta B = \frac{x(z^2 - 1)}{(1 + xz)(x + z)}$$

in which $x = \sqrt{k'_1 k'_2}/H$ and $z = \sqrt{k'_2}/\sqrt{k'_1}$. The maximum value of ΔB is

$$(18) \quad \Delta B_{\max.} = \frac{z - 1}{z + 1}$$

and occurs at $\text{pH} = (\text{pk}'_2 + \text{pk}'_1)/2$. The shift in the total amount of base bound by a complex molecule containing a num-

ber of acid radicals is the algebraic sum of the shifts in the amounts of base bound by each. On this basis we have determined values of k' for the two histidines assumed to interact with each heme which account for the ΔB -pH data for 25° in a very satisfactory way. The smooth curve of Fig. 2 is constructed for the case in which the pK' of one histidine is changed from 7.81 to 6.80, that of the other from 5.25 to 5.75, as a result of oxygenation of the associated heme. The circles give the experimental data taken from an earlier study (6). Over most of the range the agreement is good. The discrepancy at the extreme alkaline

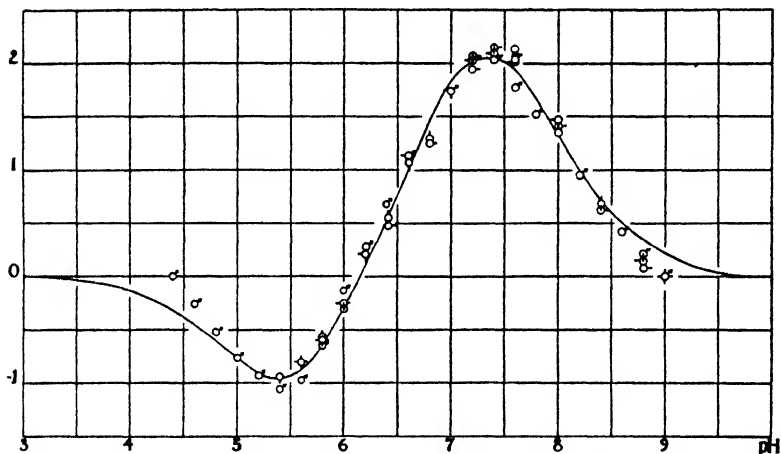


FIG. 2. Difference in the number of equivalents of base bound per mole by oxygenated and reduced hemoglobin as a function of pH. The smooth curve is calculated on the basis of assumptions discussed in the text.

end of the curve might be attributed to the difficulty of completely reducing hemoglobin in this region; that at the other end to the rapidity with which hemoglobin undergoes a reversible change involving loss of oxygen-combining power at strongly acid reactions. It is found that altering the value of $pK'_2 - pK'_1$ for either histidine unit by as much as 10 per cent very noticeably impairs the fit of the curves, as does altering the mean pK' value of either group by 0.1 to 0.2 unit. Were we to choose different pK' values for the histidines associated with different hemes, the effect would be to broaden the curve and decrease the fit. On the other hand,

very little improvement could be effected by assuming that more than two histidines interact with each heme, although if the number were made sufficiently large some compression of the curves without loss of amplitude could be achieved. On the whole, therefore, it appears that our assumption is justified and that just two histidine groups interact with each heme in accordance with the pK' values given above. The elements of the picture then fit together in a very satisfactory way, and a variety of facts involving the heats of oxygenation and the amount of base bound all find a common explanation in the view that the hemoglobin molecule is composed of four identical histidine-heme complexes. In particular it may be pointed out that the pK' values ascribable to the two histidine groups in the oxygenated complex conform to the limits predicted from the effect of temperature on the titration curve of oxyhemoglobin.

We have purposely analyzed the situation on the most general basis. The result at which we have arrived, however, accords closely with the model recently proposed by Pauling (4) on the basis of which he has been able to account for the oxygen dissociation curves of hemoglobin very exactly. In this model, to be sure, histidine finds no place, but it is assumed that the four hemes are located at the corners of a square and that they are all identical in respect to their oxygen affinity except in so far as the equilibrium constant of each is affected by the oxygenation of the adjacent hemes. It is of interest to compare the free energy of interaction of two adjacent hemes as given by this model with the free energies of interaction of each heme with the two associated histidine units. The value for the heme-heme interaction is 1470 calories; the values for the heme-histidine interactions, calculated from the pK' values given above, are 680 and 1380 calories. They are of the same general magnitude. Our results as they relate to the interaction of each heme with two histidine groups also accord with Conant's (8) picture of the hemoglobin molecule, although of course they do not of themselves imply anything so specific. In this picture it is assumed that the ferrous iron of each heme is coordinated with the 4 nitrogen atoms of the porphyrin ring in one plane and with one group of the globin part of the molecule above, and one below, this plane. When the heme is oxygenated, it is supposed that one of the two globin

groups is displaced from the iron by a molecule of oxygen, although the group still remains attached to the hemoglobin molecule through the globin. Provided therefore we identify these two globin groups with two histidines, we have a physical picture which would account satisfactorily for our own conclusions. The dissociation of the imidazole groups of both histidines would certainly be affected by the introduction of oxygen into the heme. It is known that the dissociation constant of the imidazole group is very sensitive to substituents and may be either increased or decreased, depending on the nature of the substituent. Thus Kirby and Neuberger (9) have shown that the acidity constant of glyoxaline is raised from 6.95 to 7.86 by the introduction of a methyl group in the 2 position and still further to 8.36 by an additional methyl group in the 4 position. On the other hand the introduction of a phenyl group in the 4 (or 5) position lowers the constant to 6.00, which is about the value observed in histidine. Of the two constants which we have ascribed to the histidines in reduced (deoxygenated) hemoglobin, one ($pK' = 5.25$) is much lower, the other ($pK' = 7.81$) much higher, than that of free histidine. This is perhaps not surprising if we suppose that the two histidines are situated on opposite sides of the plane of the porphyrin ring. When oxygenation occurs, the two values are shifted in opposite directions, each towards the value characteristic of free histidine. It may be remarked that as compared with the interaction of the hemes and the histidines, the interaction of the different hemes, as required by Pauling's model, presents a more difficult problem in relation to the spatial arrangement of these large groups.

Let us return now to the problem of the heat oxygenation. We have seen that this heat changes over the pH range in accordance with the product $\Delta B \times 6500$ calories. Probably the best values of ΔB are those calculated from the four pK' values given above, and it is these values which we have employed in calculating $Q_s - Q_o$. From the results so obtained we may at once reckon the actual heat of oxygenation over the whole pH range from a knowledge of its value at any one pH. Of Roughton's two directly determined values for the heat absorbed by combination of hemoglobin with 1 mole of oxygen, that of -9350 calories for pH 6.8 is the more reliable. It appears to be subject to an

uncertainty of no more than a few per cent. We have used 4 times this value as a fixed point for locating Curve 1 in Fig. 3, which gives the heat absorbed due to the combination of 1 mole of hemoglobin with 4 moles of oxygen over the range from pH 3 to 11. This leads to a value of $-46,800$ calories for Q_0 , the heat of the reaction as it occurs without an accompanying change in the amount of base bound. One-fourth of this quantity, or $11,700$

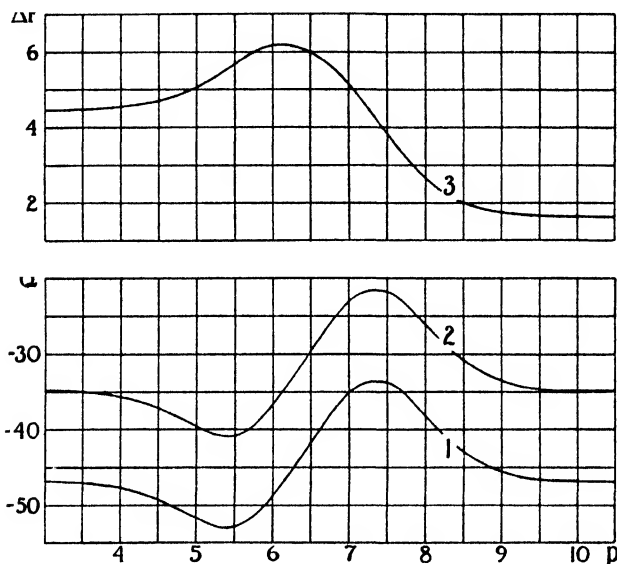


FIG. 3. Curve 1, heat absorbed owing to the reaction of 1 mole of hemoglobin with 4 moles of gaseous oxygen; Curve 2, heat absorbed owing to the reaction of 1 mole of hemoglobin with 4 moles of oxygen in solution; Curve 3, increase of free energy accompanying the reaction of 1 mole of hemoglobin with 4 moles of oxygen at a partial pressure of 1 mm. of Hg. The ordinates express kilocalories.

calories, is to be compared with Roughton's figures for pH 9.5 where there is no appreciable shift in base bound due to oxygenation. Roughton gives results for two hemoglobin preparations. For the first the directly measured heat at 19° was $12,400$ calories and the calculated heat $12,450 \pm 1200$ calories; for the second the corresponding values are $14,250$ and $14,100 \pm 1000$ calories. At this alkaline reaction measurements are more difficult and

much less accurate than at pH 6.8, and Roughton's results do not appear to be at serious variance with our calculations.

Roughton's measurements were made on ox hemoglobin, ours on hemoglobin of the horse. It might be objected that it is unjustifiable to combine results on the two kinds of hemoglobin, as we have done in using Roughton's value for the heat at pH 6.8. In view of such a possible objection Dr. D. B. Dill of the Harvard Fatigue Laboratory was good enough to determine for us oxygen dissociation curves of one of our own preparations of horse hemoglobin at different temperatures. The crystallized

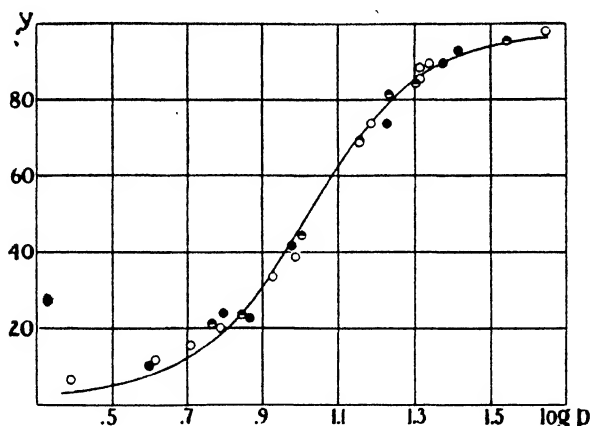


FIG. 4. Oxygen dissociation curves of horse hemoglobin obtained by Dill at different temperatures: \circ , 20°; \bullet , 40°; \ominus , 0.5°. The smooth curve corresponds to Pauling's model. y represents percentage saturation of hemoglobin with oxygen.

protein was dissolved in a phosphate buffer of ionic strength 0.5 and pH 7.05² at 25° to give a solution containing 49.6 gm. of hemoglobin per liter. Oxygen dissociation curves were determined at 0.5°, 20°, and 40°. The percentages of total hemoglobin in the active form in the solutions studied at these three temperatures were 98, 96.5, and 85.5 respectively. The results of the measurements are shown in Fig. 4, in which the data for the three temperatures have been brought together by adding 0.615

² Total phosphate = 0.212 mole per liter; mole fraction Na_2HPO_4 = 0.680. See Green (10).

to the values of $\log p$ at 0.5° and subtracting 0.425 from the values at 40° . The smooth curve corresponds to Pauling's model (4). On the basis of Equation 2 these figures give for the heat absorbed due to the reaction of 1 mole of oxygen with 1 mole of hemoglobin at the mean temperatures 10° and 30° the values 11,500 and 9100 calories respectively. The average value for 20° may be taken as 10,300, with an uncertainty of about 1000 calories. Unfortunately, no measurements were made of the pH of the solutions, but since they were made by dissolving approximately isoelectric protein (pH 6.81 for reduced hemoglobin) the pH must have been somewhat acid to that of the buffer (7.05) and not far from that at which Roughton's value of 9350 was obtained. At pH 6.9 the change in the heat corresponding to a change in pH of 0.1 unit amounts to about 550 calories. These results are therefore in agreement with the more extensive studies of Roughton on ox hemoglobin.³

In Equation 1 the activity of the oxygen is taken as equal to the partial pressure of oxygen in the gas phase. The standard state is therefore that of unit partial pressure, and the heats that we have calculated are for the reaction of hemoglobin in solution with oxygen at unit partial pressure in the gas phase. These heats therefore include the heat of solution of oxygen in the liquid phase, which has nothing to do with the combination of oxygen with hemoglobin. It is of interest to consider how much of the calculated heat, *e.g.* 9350 calories at pH 6.8, is due to this heat of solution of oxygen. The heat of solution of oxygen in pure water may be calculated from the data on the solubility of oxygen in water as a function of temperature. On the basis of the data given in the handbooks we obtain the following values for the heat absorbed (in calories) due to the solution of 1 mole of oxygen: at 10° , -3620 ; at 20° , -3200 ; at 30° , -2940 . These figures are

³ In calculating the heat of oxygenation by the van't Hoff equation Roughton has for some reason reduced the dissociation pressures observed at different temperatures to 0° (273° absolute). The correct value of the heat is obtained by using the actual pressures observed at each temperature, since the standard state for the oxygen gas must be defined by the same hydrostatic pressure at each temperature. Actually the effect of Roughton's procedure does not appear to make a significant difference in the value for the heat at 25° . Values calculated directly from Roughton's scale factors ((2), p. 2121) are $0-10^\circ$, 8680; $10-20^\circ$, 9600; $20-30^\circ$, 10,900; $30-40^\circ$, 8900; average 9500.

obtained by taking the activity of oxygen in solution as proportional to its molality; in other words by treating the solutions as ideal solutions. In so far as this is true the values should be independent both of the pressure of the saturating gas and the concentration of the oxygen in solution. They show that of the 9350 calories about 3000, or approximately one-third, are due simply to the heat of solution of oxygen at 25°. Curve 2 of Fig. 3 is for the heat of reaction of hemoglobin with oxygen in solution, obtained by taking account of this effect.

In contrast to this the free energy of the reaction is of course independent of whether the oxygen is in solution or in the gaseous phase at the corresponding partial pressure. Let us consider the free energy change in detail. If we denote by ΔF the increase of free energy accompanying the combination of 1 mole of hemoglobin with 4 moles of oxygen when the reactants are in their standard states, then

$$(19) \quad \Delta F = -RT \ln L_4^4$$

Equations 3 and 13 therefore make it possible, from a knowledge of ΔB in relation to pH , to reckon the change of ΔF with pH , just as we have reckoned the change in the heat of the reaction with pH . If ΔF_1 and ΔF_2 be the values of ΔF at pH_1 and pH_2 respectively, then

$$(20) \quad \Delta F_2 - \Delta F_1 = -RT (\ln L_4^4)_{pH_2} + RT^2 (\ln L_4^4)_{pH_1} \\ - 2.303 RT \int_{pH_1}^{pH_2} \Delta B \, dpH$$

If, as in reckoning the heat of the reaction, we use the values of ΔB given by the apparent dissociation constants discussed above, this equation may be integrated directly to give, for 25°

$$(21) \quad \Delta F_2 - \Delta F_1 = -4 \times 1365 \log \frac{H + k'_2}{H + k'_1} \cdot \frac{H + k'_4}{H + k'_3} \text{ calories}$$

in which the four k 's are given by $pk'_1 = 7.81$, $pk'_2 = 6.80$, $pk'_3 = 5.25$, and $pk'_4 = 5.75$. From a knowledge of ΔF at any one pH , we may reckon it for the whole pH range. Pauling has shown that all the data of Ferry and Green at 25° when reduced to pH 8.30 may be accurately fitted by a choice of constants which leads to a value of $\Delta F = +2200$ calories for this pH . Curve 3 in Fig. 3 is plotted from Equation 21 on the basis of this value.

The value 2200 is for the reaction when the oxygen is in the standard state; *i.e.*, at a partial pressure of 1 mm. For any other pressure p mm. ΔF will be $(2200 - 5450 \log p)$ calories. It follows from Equation 3 that the change with pH in the free energy of each stage of the oxygenation process is the same and equal to $\frac{1}{4} \partial \Delta F / \partial \text{pH}$, although, as Pauling shows, the free energy itself is different for each stage of the process, owing to the interaction of the hemes.

SUMMARY

The variation in the heat of oxygenation of hemoglobin with pH has been studied from pH 3 to 11. The results can be accounted for on the basis of the dissociation of hydrogen ion which accompanies oxygenation if we assume a heat of dissociation of 6500 calories per equivalent. This is the heat of dissociation characteristic of the imidazole group of histidine, and indicates that it is groups of this kind which dissociate as a result of oxygenation of the hemoglobin molecule. From these results and Roughton's value for the heat of oxygenation at pH 6.8 we have calculated the heat of oxygenation of hemoglobin over the whole pH range from 3 to 11. It is pointed out that the heat is the same for each stage of the oxygenation process, as is the shift in the amount of base bound at constant pH. A further analysis based on the shift in the amount of base bound at 25° indicates that each molecule of hemoglobin contains four identical heme-histidine complexes, in which two histidine units interact with each heme, the energy of interaction amounting to 680 calories for one and to 1380 calories for the other. These conclusions accord with inferences based on the effect of temperature on the dissociation curves of oxyhemoglobin, and they also agree with a model recently proposed by Pauling. Finally on the basis of the shift in base bound as a function of pH we have calculated the free energy of oxygenation from pH 3 to 11.

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APPLICATIONS OF DIPHENYLAMINE IN THE DETERMINATION OF LEVULOSE IN BIOLOGICAL MEDIA

I. THE DETERMINATION OF INULIN

II. THE DETERMINATION OF LEVULOSE IN SMALL AMOUNTS OF BLOOD

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The Determination of Inulin

The formation of a blue compound of diphenylamine and levulose in the presence of concentrated HCl was applied semi-quantitatively to urine by Jolles (1) and quantitatively to blood filtrates by van Creveld (2), whose method for blood levulose has since been variously modified (3-7). In the van Creveld method and its modifications, the reaction was carried out in an aqueous medium and the water-insoluble blue product extracted into an organic solvent for color comparison. This disadvantage was removed by Herbert (8) by the addition of sufficient alcohol to keep both diphenylamine and colored product in solution during and after the reaction. The method here described is an application of Herbert's method to the determination of inulin, the final determination being made in the single cell, compensating photoelectric colorimeter described by Evelyn (9). Glucose and levulose present in the material tested are removed by fermentation with yeast. The inulin is hydrolyzed by the strong acid in which the reaction occurs.

Reagents—

1. Precipitating media (10). (a) 1.25 per cent $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, in 0.03125 N H_2SO_4 ; (b) 0.75 N NaOH.

Although these concentrations of reagents are those recommended by Somogyi (10) for whole blood rather than plasma,

they have been found quite satisfactory and are employed because of their general use in this laboratory. The weaker reagents recommended for plasma may be substituted without affecting this method.

2. Stock diphenylamine solution. 20 per cent diphenylamine in alcohol.

The stock solution is kept in a cool place in a dark bottle. Diphenylamine which precipitates out at ice box temperatures is easily redissolved as the solution is brought to room temperature. The diphenylamine used was obtained from the Eastman Kodak Company.

3. Acid alcohol-diphenylamine reagent. 95 per cent ethyl alcohol, 70 parts; concentrated HCl, 50 parts; stock 20 per cent diphenylamine solution, 6 parts.

This solution is also kept in a cool place in dark bottles. Although it is stable for at least 1 month (8), we have prepared it weekly.

4. Thoroughly washed bakers' yeast.

Bakers' yeast (obtainable from Standard Brands, Inc.) is washed by centrifugation with distilled water until the supernatant is clear for two washings.

Apparatus—

1. Water bath with removable tube rack.

2. Tall resistance glass test-tubes graduated at 25 cc.

3. Photoelectric colorimeter of the type described by Evelyn (9), equipped with a light filter transmitting at a maximum of about 620 $m\mu$.¹

Procedure

Serum or Plasma Inulin—(a) 1 part of serum or plasma is added to 8 parts of $ZnSO_4$ solution, mixed, and the protein precipitated by addition of 1 part of 0.75 N NaOH. The volume of filtrate obtained from small samples (0.5 or 1.0 cc.) of plasma or serum may be increased by centrifuging the mixture before filtration. After filtration, the protein-free filtrate is placed in a centrifuge tube which contains about $\frac{1}{2}$ volume of thoroughly

¹ The photoelectric colorimeter, light filters, and absorption tubes may be obtained from the Rubicon Company, Philadelphia.

packed yeast. These tubes are prepared by adding the required volume of 1:1 suspension of washed yeast in water to a centrifuge tube, centrifuging at 2800 R.P.M. for 20 minutes, pouring off the supernatant, and allowing the tubes to dry during inversion in a rack. The water is then removed from the lips of the tubes, and droplets which may have clung to the tube wall removed with clean gauze.

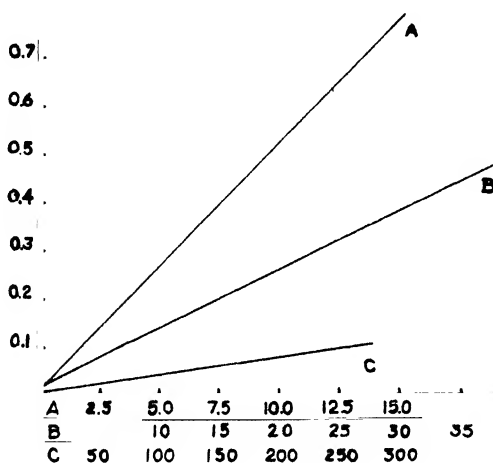


FIG. 1. The ordinate represents color density (*L* value (9)); abscissa: Curve A, inulin determination, levulose equivalent in mg. per 100 cc. of protein-free filtrate or water; Curve B, levulose determination, levulose in mg. per 100 cc. of blood; Curve C, levulose determination of glucose equivalent in mg. per 100 cc. of blood.

(b) The yeast is stirred into the plasma filtrate with a clean dry rod and the mixture allowed to stand with occasional shaking for 15 to 30 minutes. The tubes are then centrifuged at the rate and for the length of time used in packing the yeast from its water suspension. The supernatant fluid is then decanted through small filter papers.

(c) A 1 cc. portion of filtrate is transferred to a tall test-tube graduated at 25 cc., or, if deposits from the water bath can be excluded, into a colorimeter absorption tube (8) which has been graduated at this mark. The sides of the tube are washed down

during the addition of 5 cc. of acid alcohol-diphenylamine reagent and the tubes at once placed in a boiling water bath for exactly 15 minutes. The rack of tubes is then hastily removed from the water bath and cooled in an ice bath for about 5 minutes, at which time they are made up to 25 cc. with alcohol. The compensating blank is prepared from an identically treated sample of plasma or serum taken before the administration of inulin.

(d) The color comparison is made against the compensating blank with Filter 620 (transmission 595 to 660 $m\mu$). The inulin content is found by reference to a calibration chart which has been prepared from samples of pure levulose in precipitating media which have been treated exactly as described in the preceding paragraph (c) (Fig. 1, Curve A).

Urine Inulin—Urine inulin may be determined by this method when present in a concentration of about 2.5 per cent. The urine sample, or the mixture of urine diluted in bladder washings, is diluted in distilled water to at least 1000-fold of the original urine. Although urine protein in concentrations as high as 5 per cent does not interfere with the reaction at dilutions higher than 1 part in 500, it may be removed before dilution by treatment of the urine with the precipitating media described above. The diluted urine sample is treated exactly as the blood filtrates in paragraphs (b), (c), and (d) above, with the exception that the compensating blank is prepared from the distilled water in which the urine was diluted.

Results

The factors affecting the reaction are as follows:

Duration of Heating—At 15 minutes the color obtained is equivalent to 88 per cent of that obtained at 30 minutes of heating. The shorter period has been used because the color developed follows Beer's law throughout the range equivalent to 2.5 to about 15.0 mg. per cent of material tested. The deeper color obtained with longer heating shows a deviation from linearity at lower concentrations. Because the maximum color has not been developed by the shorter period of heating, care must be taken to avoid prolonged contact of reagent and sample before heating and to cool the tubes immediately and thoroughly at the end of heating.

Chromogen Present in Blood Plasma—The chromogen remaining in plasma filtrates after treatment with yeast amounts to the equivalent of about 1 mg. per 100 cc. of plasma inulin and is canceled in the determination by the use of the compensating blank prepared from plasma obtained before the injection of inulin.

Chromogens Present in Urine—Nitrates and nitrites yield blue colors with this reagent. Consequently, the reaction cannot be attempted in undiluted urine, although dilution to 1 part in 1000 or more practically abolishes the color obtained from the normal dog or human urine. There is no perceptible blank obtained from urine to which nitrites have been added to a concentration of 1 part in 1000 or nitrate to a concentration of 1 part in 100, the samples subsequently having been diluted a thousand times. These concentrations of urine nitrate and nitrite will rarely be reached in the urine of the intact animal.

Recovery—The levulose used in the preparation of the calibration chart (Fig. 1, Curves A and B) had a specific rotation of -91.5° at 20° (average of two determinations by different observers).² The inulin used (Pfanstiehl, c.p.) was dissolved in water by heat, passed through a Seitz filter, thrice reprecipitated from water solution with alcohol, and carefully dried in a vacuum desiccator over CaCl_2 . The recovery of inulin from blood plasma and from solutions in water and precipitating medium averaged 99 per cent by weight and varied from 98 to 101 per cent at concentrations equivalent to 5 to 15 mg. per 100 cc. of plasma filtrate, precipitating medium, or water (average of 60 determinations).

The same inulin sample was found to have a levulose equivalent of 94 per cent by weight after acid hydrolysis and determination of hydrolyzable reducing substance by the Shaffer-Somogyi method (Reagent 50, 5 gm. of KI, 150 cc. of 0.1 N iodate per liter) (11). Hydrolysis was carried out by addition of 0.2 cc. of 1 N H_2SO_4 to 5 cc. of inulin solution; the tubes were heated in a boiling water bath for 15 minutes and the reducing substance determined after neutralization. An 8-fold increase in the concentration of acid did not increase the yield of reducing substance. The whole of the reducing substance thus obtained was fermented by yeast.

² The writers wish to acknowledge their thanks to Dr. E. C. Kleiderer and Dr. E. J. Hughes of the Research Division of Eli Lilly and Company for these analyses.

Treatment of the fermented hydrolysate with diphenylamine reagent gave a color equivalent to from 5 to 7 per cent of the inulin used (three determinations). This chromogen, as determined by the average difference of the two methods, was equal to 6 per cent by weight of the inulin sample and is apparently identical with the fructose anhydride described by Jackson and Goergen (12), which amounted to 5 per cent of their inulin sample. Our recovery of 99 per cent inulin by weight suggests that the inulin used had a non-chromogenic aldose content of about 1 per cent, and thus that the hydrolyzable levulose content of our inulin was 93 per cent, a value which agrees well with recoveries of 92 and 94 per cent reported in the literature (12, 13).

SUMMARY

A photolorimetric method for the determination of small amounts of inulin in blood and urine is described. The method depends upon the color produced by levulose and probably by levulose anhydrides in contact with strongly acid diphenylamine.

The Determination of Levulose in Small Amounts of Blood

The method here described is a convenient modification of Herbert's method (8) which requires only 0.2 cc. of blood.

Reagents—

1. Precipitating media (modified from Hagedorn and Jensen (14)). (a) 4.5 per cent $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; (b) 0.2 N NaOH.
2. Stock diphenylamine solution.
3. Acid alcohol-diphenylamine reagent.

Apparatus—

1. Resistance glass test-tubes graduated at 10 cc.
2. Water bath, tube rack, and photoelectric colorimeter.

Procedure

(a) *Precipitation of Blood Protein*—0.2 cc. of blood is delivered from a pipette calibrated "to contain" into a centrifuge tube containing 1 cc. of ZnSO_4 solution and the pipette rinsed back into the tube. 1 cc. of 0.2 N NaOH is added and the tube placed in a boiling water bath for 4 minutes, when it is removed and centrifuged at about 2800 R.P.M. for 5 minutes.

(b) *Development of Color*—1 cc. of the supernatant fluid is taken

into a pipette whose tip is guarded by a wisp of washed cotton. The cotton is removed and the protein-free filtrate transferred to a test-tube graduated at 10 cc., or, if deposits from the water bath can be excluded, to a colorimeter absorption tube graduated at this mark. 5 cc. of acid alcohol-diphenylamine reagent are added and the tubes at once placed in a boiling water bath for exactly 15 minutes. When the tube is removed from the water bath, it is cooled in an ice bath for about 5 minutes and made up to 10 cc. with alcohol. The compensating blank is prepared from an identically treated sample of blood taken before administration of levulose.

(c) *Color Determination*—Color comparison is made with reference to the compensating blank with Filter 620. The levulose content is found by reference to a calibration chart prepared from samples of pure levulose in precipitating media treated exactly as in paragraphs (b) and (c), p. 603, (Fig. 1, Curve B).

Results

Effect of Blood Glucose—The color given by solutions of glucose and reagent heated for 15 minutes varies slightly in its levulose equivalent. The average color obtained in terms of blood glucose is represented in Fig. 1, Curve C, in which 100 mg. per 100 cc. of blood glucose are equivalent to about 2.5 mg. per cent of levulose. Error from this source is abolished by the compensating blank when blood glucose is constant, and the error amounts to only 0.5 mg. of levulose per 100 cc. with glucose concentrations varying 20 mg. per 100 cc. When there is reason to suspect that wider variations will occur, total blood sugar may be separately determined by some micromethod. A close approximation of true levulose may then be made by subtraction from total blood sugar of the apparent levulose to yield approximate true glucose. The levulose equivalent of the approximate blood glucose is found by reference to the calibration chart (Fig. 1, Curve C) and this value applied to correct the apparent blood levulose.

The recovery of levulose added to blood averages 100 per cent at concentrations varying from 5 to 50 mg. per 100 cc. of blood. The range of error amounts to about 0.5 mg. of levulose per 100 cc. (average of thirty determinations).

The method is applicable to the study of levulose metabolism

in small animals and has been satisfactorily used in the clinical determination of levulose tolerance.

SUMMARY

A method is presented for the determination of levulose in 0.2 cc. of blood.

The authors wish to acknowledge their gratitude to the John and Mary R. Markle Foundation for a grant to one of us (A. C. C.).

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A DIRECT COLORIMETRIC METHOD FOR THE DETERMINATION OF INULIN IN BLOOD AND URINE*

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The renal clearance of inulin was introduced almost simultaneously several years ago by Richards, Westfall, and Bott (1) and by Shannon and Smith (2) for the measurement of glomerular filtration rate. Since then the inulin clearance has been employed by a number of investigators, especially for studies on the human kidney. However, because of the large quantities of inulin that must be administered for the clearance test, and the insensitive and tedious methods of analysis employed, this clearance has not found the favor that it merits.

Inulin hitherto has been determined by the reducing power, as carbohydrate, of the sample before and after hydrolysis by acid. Such a method suffers from the inconveniences and errors involved in analyses performed by difference and cannot be employed for accurate determination of inulin in blood plasma at concentrations below 50 mg. per cent.

In order to perform certain studies of inulin excretion at very low plasma levels, and furthermore with the idea of simplifying the analytical technique of the clearance, and of reducing the quantity of inulin to be injected, we have devised the method here described. This new procedure depends on a reaction between inulin and alcoholic diphenylamine in hot acid solution. With inulin, as with fructose (van Creveld (3), Patterson (4), Herbert (5), and others), this reagent gives an intense, clear blue color. The reagent reacts only slightly with glucose. When glucose and fructose are removed by fermentation with yeast, inulin may be determined specifically in biological fluids by this method.

* This work has been aided by a grant from the Douglas Smith Foundation for Medical Research.

Our method gives very precise results in the determination of plasma or urine filtrates containing 4 micrograms or more of inulin per cc., and it also yields satisfactory values on plasma filtrates containing as small an amount as 2 micrograms per cc.

Reagents—

Diphenylamine reagent. 10.00 gm. of reagent grade diphenylamine (Merck, m.p. 52.5–53.3°) are dissolved in redistilled absolute ethyl alcohol and diluted to a volume of 100 cc. with the alcohol. This solution must be kept in a dark bottle. Of this alcoholic diphenylamine solution 12 volumes are added to 192 volumes of a mixture which contains 80 volumes of c.p. concentrated HCl and 112 volumes of redistilled absolute ethyl alcohol. If kept in a dark bottle, this reagent may be used for 1 week; the very faint color which develops during this time does not interfere with the method.

Acid cadmium sulfate solution. 13.0 gm. of reagent grade cadmium sulfate ($3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$) and 63.5 cc. of exactly 1 N sulfuric acid are made to 1 liter with distilled water.

1.10 N sodium hydroxide. Reagent grade sodium hydroxide "from sodium" is used for this solution.

Procedure

Removal of Fermentable Carbohydrate—Because glucose reacts slightly with the diphenylamine reagent, it must be removed when very accurate determinations of inulin are required at values below 30 mg. per 100 cc. of plasma, or whenever analyses are made on samples from subjects having hyperglycemia or glycosuria. The glucose is removed as follows: To 5 or 10 cc. of plasma or serum in a 15 cc. centrifuge tube is added 0.5 or 1.0 cc. respectively of a suspension of yeast cells.¹ The tube is shaken and incubated at 38° for 30 minutes, and then centrifuged at high speed for 15 minutes. At the same time the volume of yeast cells is obtained in the Wintrobe hematocrit tube (6). The supernatant plasma

¹ The yeast suspension is prepared by washing about 50 gm. of starch-free yeast in 100 cc. of distilled water. The water suspension is centrifuged at low speed and the supernatant liquid decanted. This procedure is repeated five times. The washed yeast cells can be kept in a stoppered container in the refrigerator for 2 weeks. The cells should be washed again once immediately before use.

is deproteinized as described below. Whenever the yeast fermentation technique is employed, it should be performed as above; if used directly on filtrates, it yields turbid solutions which interfere with the final estimation of the color.

Precipitation of Blood or Urine Protein—The method of Fujita and Iwatake (7) is employed. To 1 volume of plasma, serum, or urine are added 8 volumes of the acid cadmium reagent, followed by 1 volume of 1.10 N sodium hydroxide. The mixture is agitated gently while the sodium hydroxide is being added, and is then shaken vigorously for a few seconds. After standing 15 minutes the mixture is filtered and the filtrate diluted, if necessary, to contain between 2 and 12 micrograms of inulin per cc.

Development of Color—The reaction is performed in a heavy walled test-tube which measures approximately 20×110 mm. These tubes must be capable of withstanding considerable changes of temperature and pressure, and also must have covers which make a perfectly tight fit to prevent evaporation during heating. We have employed a special tube for this purpose.²

5 cc. of diluted urine or filtrate of plasma or serum are placed in one of the glass tubes and 10 cc. of the diphenylamine reagent added. The tube is agitated to dissolve the precipitate, and to obtain complete mixing of the aqueous and alcoholic solutions. The screw-cap is sealed tightly onto the tube which is placed in a bath containing vigorously boiling water. It is convenient to use a rack capable of holding eighteen tubes. The temperature of the bath should be uniform throughout, and there should be provision for free circulation of water under and around the individual tubes. After the tube has been heated in the boiling water for exactly 60 minutes, it is removed and immediately plunged into water and cooled to room temperature. The cooled solution is kept at room temperature for 10 minutes longer and then transferred as described below.

Caution—Care should be taken to protect oneself against the possibility of a defective tube exploding during the boiling or sudden cooling.

Estimation of Color Intensity—We find that the Evelyn photo-

² Round bottom tubes complete with screw-caps may be obtained from E. H. Sargent and Company, Chicago. Rubber liners must be heated in 10 per cent sodium hydroxide before they are used for the first time.

electric colorimeter (8) gives very precise, reproducible results with a minimum time of reading. The solution is transferred to one of the "S" test-tubes furnished with the instrument and the light transmission determined with a No. 660 filter.³ The equivalent inulin concentration is determined from a calibration curve which relates per cent light transmission to concentration of inulin.

Undoubtedly other types of instruments can be employed satisfactorily. We have used the Zeiss Pulfrich stufenphotometer with good results; however, this instrument is less satisfactory than the Evelyn photoelectric colorimeter. For inulin concentrations above 5 mg. per cent the Duboscq colorimeter gives results which are very satisfactory.

Determination of Blank Value of Reagents—5 cc. of distilled water are heated with the diphenylamine reagent for 1 hour, and cooled as above. The final color yielded by the blank reagents should be very slight. It is subtracted from the total color by adjusting the photoelectric colorimeter to read 100 per cent transmission with the tube containing the blank. The corresponding unknowns are then read at the "center setting" of the blank. If the blank solutions show slight variations from tube to tube, several of them should be combined and used for an average setting.

Calibration—Inulin (c.p. Pfanstiehl) is dried at 80° to 100° for 1 hour and cooled in a desiccator. A solution is prepared containing exactly 1 gm. of this dried inulin in 100 cc. of water. From this solution dilute standards containing from 0.001 to 0.015 mg. per cc. are prepared, and these are analyzed by the method given above beginning with "Development of color." The logarithm of the per cent light transmission as obtained in the Evelyn colorimeter, or the stufenphotometer, is plotted against concentration of inulin. The calibration line plotted on semilogarithm paper is shown in Fig. 1 where it may be seen that the color intensity exhibits a linear relationship to inulin concentration from 0.001 to 0.005 mg. per cc. At higher concentrations the calibration line is curvilinear, but is always reproducible. *Because inulin varies slightly in composition, depending upon its source, calibration curves should be made on the particular inulin used by each laboratory.*

³ The colorimeter and filters may be obtained from the Rubicon Company, Philadelphia.

Calculations and Corrections for Non-Inulin Chromogenic Substances in Blood and Urine—For aqueous solutions or very dilute urine containing inulin the concentration is obtained directly from the calibration line, or when the ordinary colorimeter is employed, by comparison with standard inulin solutions. However, for serum or plasma a correction must be made for the color

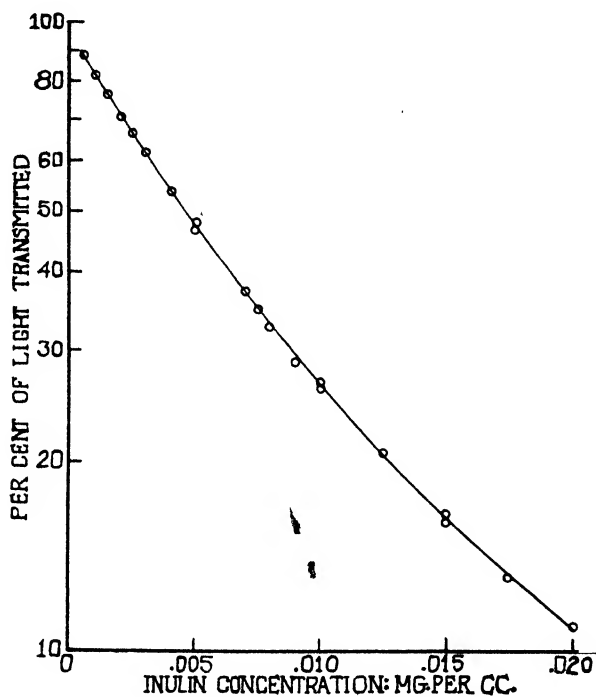


FIG. 1. Calibration curve showing relation between the per cent of light transmitted and the concentration of inulin. (Evelyn photoelectric colorimeter with Filter 660.)

produced by the non-inulin chromogenic compounds in the blood. This is accomplished by one of the following methods.

Method 1 (When Highest Accuracy Is Required)—A sample of plasma or serum is obtained shortly before the injection of inulin. It is treated by the yeast fermentation technique and the inulin equivalent of the residual chromogenic material ob-

tained. This value is so small and so constant that it may be subtracted from the total inulin value of all blood specimens taken after injection of inulin. In most cases it should be sufficient to determine this value once on a control blood under the conditions of the particular experiment and to use it as a constant correction thereafter.

The dilution effect from the water in the yeast suspension is obtained by applying the hematocrit value obtained on the yeast suspension.

For concentrated urine containing small amounts of inulin the urine itself may contribute a small blank. In such cases the blank is obtained on a specimen of urine collected under the same conditions as the subsequent ones containing inulin. The correction due to non-inulin chromogenic material is very small in almost all inulin clearance experiments. It might be large when other substances such as glucose are injected with the inulin; in such a contingency the fermentation technique described for the plasma or serum should also be applied to the urine.

Method 2 (When Fermentation Is Not Employed)—The plasma or serum obtained before the injection of inulin is analyzed for non-inulin chromogenic material. The blank value averages about 7 mg. per cent. This value is subtracted from the total inulin values of the subsequent samples. Unless the inulin concentration lies above 30 mg. per cent, this method leads to fairly large errors.

EXPERIMENTAL

Determination of Inulin Added to Normal Human Plasma and Urine—Inulin was added to human serum or plasma and analyzed by the complete procedure including fermentation. The results given in Table I show the high degree of accuracy obtained by the method. From 4 to 15 mg. per 100 cc. the error is 2 per cent or less; and even at the exceedingly low value of 2 mg. per 100 cc. the error is only 5 per cent. Comparable recoveries are obtained with analyses performed on normal urine containing added inulin, as shown in Table II.

Determination of Inulin Added to Abnormal Human Plasma and Urine—Inulin was added to plasma and urine obtained from

patients suffering from severe uremia or severe jaundice with high serum and urine bilirubin contents. Also, urines containing large quantities of protein were studied. In each case the accuracy of the inulin determination compares favorably with the results found with normal plasma and urine.

When plasma and urine obtained from diabetic patients are both analyzed by the fermentation technique, the results agree

TABLE I
Recoveries of Inulin Added to Blood Plasma (Plasma Fermented)

Inulin added	Inulin recovered	Error
<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>per cent</i>
2.03	1.93	-4.9
4.09	4.09	0.0
8.06	8.24	+2.2
8.06	8.11	-0.6
11.55	11.40	-0.9
10.20	10.30	+1.0
14.70	14.95	+2.0

TABLE II
Recoveries of Inulin Added to Untreated Urine

Inulin added	Inulin recovered	Error
<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>per cent</i>
96.0	93.6	-2.5
96.0	96.6	+0.6
96.0	92.3	-3.9
229.0	227.6	-0.6
229.0	227.6	-0.6
229.0	221.0	-3.6

with those from normal subjects. In diabetic patients, however, the fermentation technique must always be employed.

Effect of Various Protein Precipitants—Various protein precipitants have been tested. Tungstic acid is unsuitable because a precipitate develops during heating. Trichloroacetic acid, iron, and mercury yield higher blanks for the non-inulin chromogenic substances in the blood. Somogyi zinc precipitation yields results

identical with those obtained by the cadmium precipitation described above.⁴ The cadmium precipitation was adopted in preference to the Somogyi precipitation, because the former method allows simultaneous determination of creatinine and inulin clearances. Creatinine analyses are more accurate when performed on cadmium filtrates (Miller and Allinson).⁵

Choice of Serum or Plasma for Analysis; Effect of Blood Anti-coagulant—Determinations of blood inulin yield identical values with either plasma or serum. Potassium oxalate or heparin, the only anticoagulants tested, does not interfere with the analysis.

SUMMARY

A method is described for the determination of inulin by a simple, direct colorimetric procedure which possesses great sensitivity and specificity. In this method the color reaction between fructose and diphenylamine has been adapted to the estimation of inulin in serum, plasma, and urine. The intense blue color produced by inulin is measured by a photoelectric or visual colorimeter.

By this procedure plasma inulin concentrations as low as 2 mg. per cent may be determined with a high degree of accuracy.

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⁴ In a recent paper Steinitz (9) objects to the use of cadmium hydroxide as a protein precipitant for the determination of inulin. He states that cadmium yields lower values than precipitation by zinc hydroxide. We have recently performed additional recovery experiments on serum containing 15 and 150 mg. per cent of added inulin, and have found that both types of precipitants give identical and correct values.

⁵ Miller, B. F., and Allinson, M. J. C., unpublished results.

AN APPARATUS FOR THE RAPID AND ACCURATE DETERMINATION OF LOW OSMOTIC PRESSURES

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(Received for publication, November 28, 1938)

Some of the difficulties inherent in the determination of osmotic pressure by the methods already in use were felt most acutely in this laboratory when attempts were made to study human serum fractions both in normal and in nephrotic subjects.

In the technique used so successfully by Adair (1), a collodion bag containing the protein solution is tied to the lower end of a glass tube and immersed in a vessel containing the outer fluid. Several cc. of solution, a cold room, and much time are required (equilibrium being usually reached only after a week or two). The protein concentration must be measured in each bag after the end of the experiment, since some change in concentration always occurs. A determination of the specific gravity of the fluid and a correction for capillarity are also required. These complications render the accurate determination of low pressures rather hazardous.

For clinical purposes, many investigators, following Govaerts (2) and Krogh and Nakazawa (3), have used a different system, which involves a flat membrane (usually cellophane) and requires only a fraction of a cc. of solution. Two features of the method are that a counterpressure is applied on the inner fluid through a water manometer, and that the outer fluid is usually represented only by a moist disk of filter paper. We soon realized that this method, which is suitable for approximate clinical work, becomes useless if accuracy in the determination of low pressures is desired.

In more recent years, Oakley (4) has attempted to obviate some of the defects pointed out above. Unfortunately, his apparatus requires large amounts of material, and is very sensitive to temperature changes.

The method outlined below requires no more than 0.2 cc. of solution. At room temperature equilibrium is usually reached in 3 to 4 hours, and the final reading can be made from 3 to 4 hours later. There is no determination of concentration after the reading, no correction for specific gravity or capillarity. It is accurate enough so that a protein solution as little concentrated as 0.025 mm still gives readings which usually check within less than 5 per cent.

Description of Apparatus

The apparatus is essentially composed of the following parts:

1. A hard rubber part. This encloses a horizontal flat membrane separating the inner chamber (below), which contains the liquid to be examined, from the upper chamber (above).

2. A Y-shaped glass tube. The lower branch of the tube is connected with the inner chamber; one of the upper branches is connected through a stop-cock with the outer chamber; the other branch bears a capillary graduated in mm., which is the manometer.

The following description should be read with the help of Fig. 1.

The part containing the membrane is similar, if considered upside down, to that described by Krogh and Nakazawa (3). It is entirely made of hard rubber. The central piece *d* encloses the inner chamber *h* whose capacity is about 0.2 cc. and which is to receive the protein solution. The upper face of *h* is represented by the membrane *g*, followed by a perforated, hard rubber disk *f*, a thick soft rubber washer *e*, and cylinder *b*. All these parts are firmly clamped together by screwing *c* over *b* and *d*. This arrangement isolates tightly the inner chamber *h* from the outer chamber *h'*, permitting no protein solution to escape into the latter; at the same time crumpling of the membrane, which inevitably happens when the latter is in direct contact with a soft rubber washer, is here prevented.

At the bottom of *d* the arm *l* of the glass tube is inserted and firmly held in place by screwing the hard rubber cap *i*. The latter is separated from *d* by a hard rubber washer *k* and a soft rubber washer *j*.

The lower arm of the glass tubing is of 0.5 mm. inner diameter. It is bent twice at right angles, and before dividing into its two branches it widens into chamber *m* (0.05 to 0.1 cc. in capacity).

Chamber *m* is so placed that its floor, when the apparatus is assembled, is at the same level as the membrane.

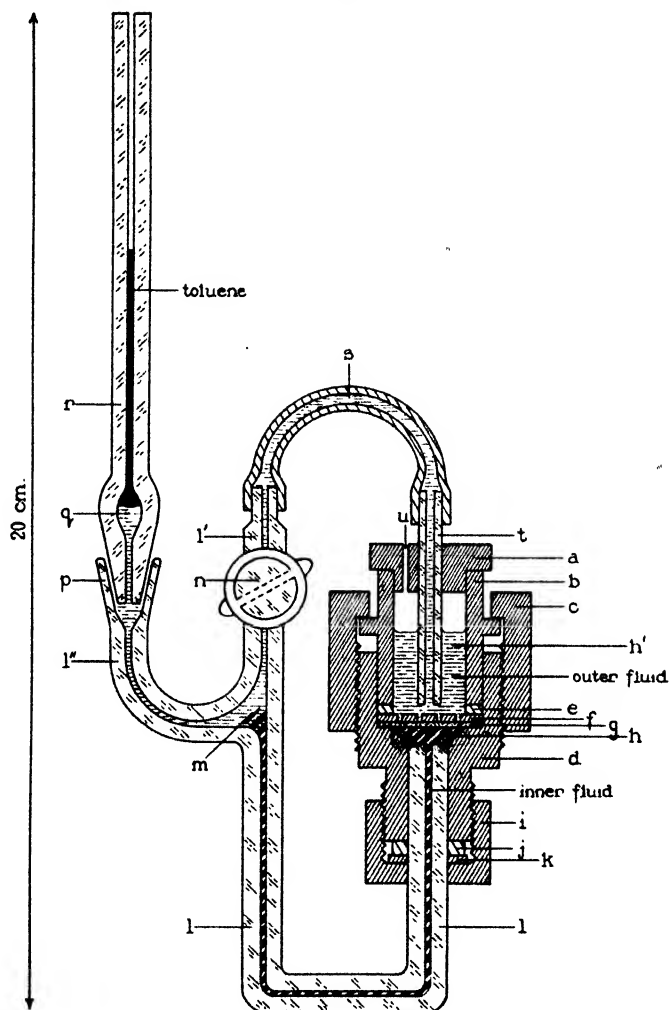


FIG. 1

One of the branches *l'* of the tube is provided with a 1-way stop-cock *n* which opens into a section of transparent rubber tubing, *s*, followed by a section of glass tubing, *t*, which passes

through the hard rubber plug *a*. The latter bears a small hole *u* which insures atmospheric pressure inside chamber *h'*.

The other branch *l''* opens into cup *p*, which bears tube *r*. The latter is made of high grade 0.2 mm. capillary tubing, and is graduated in mm. The lower part of *r* widens into chamber *q*, the capacity of which is 0.05 to 0.1 cc. Capillary *r* is firmly plugged into cup *p* and contact takes place through carefully ground surfaces.

Description of Method

The apparatus is assembled and filled in the following way.

The glass tube is first fitted to *s*, *t*, *a*, and to *i*, *k*, and *j*, but not to *r*. The membrane, which has been dipping in the solution which is to be the outer fluid, is rapidly dried between two sheets of filter paper and immediately set in place in *d*, followed by *f*, *e*, and *b*. If the membrane is very thin, it is necessary to separate it from the perforated plate by a disk of filter paper, in order to give it perfect rigidity; the diameter of the paper disk should be slightly less than the upper diameter of the inner chamber. Then *c* is firmly screwed on. The object is now inverted and about 0.2 cc. of the solution to be tested is pipetted into *h*. Arm *l* is now introduced and pressed down until the fluid fills *l* as far as the entrance to chamber *m*. Cap *i* is then tightly screwed on.

The apparatus is now inverted again and placed in a support. Chamber *h'* receives about 1.5 cc. of outer fluid, *a* is plugged in place, and a slight negative pressure is applied over cup *p* so as to bring the fluid up through *t* and *s* and down through *l'*, *m*, and *l''* until it fills cup *p*. There should be no bubbles left in the capillary parts. A small bubble at the top of *s* is immaterial.

The manometer is prepared as follows: Its lower end is dipped in toluene, which is sucked up until it fills about the lower half of chamber *q*. It is then dipped in water, which is sucked up until the toluene fills the upper half of the chamber and the beginning of the capillary. The ground surface is lubricated with vaseline, and the capillary is plugged into *p*.

The whole procedure takes but a few minutes.

The apparatus should be placed in a water bath, the water just covering the top of *c*, and in a room at approximately constant temperature. It is now ready for operation.

The stop-cock being open, the toluene column in the manometer comes into hydrostatic equilibrium with the fluid in chamber h' through l'' , l' , s , and t . The pressure thus registered is p_1 . As soon as the cock is closed the meniscus starts to rise, because to the hydrostatic pressure already present is now added the osmotic pressure which tends to transfer water through the membrane from h' to h , and therefrom to the manometer through l and l'' . The rise of toluene will finally stop when the height of the toluene balances the sum of the hydrostatic and of the osmotic pressures. The figure then read is p_2 . The osmotic pressure alone, in mm. of toluene, is $p_2 - p_1$. When, after the reading of p_2 , the cock is opened again, the toluene falls back to p_1 . Since some slight changes may

TABLE I
Osmotic Pressure of a 0.1 mm Solution, Expressed in Mm. of Toluene, at Different Temperatures

Temperature	Pressure	Temperature	Pressure
°C.	mm.	°C.	mm.
15	28.0	23	29.0
16	28.2	24	29.2
17	28.3	25	29.3
18	28.4	26	29.4
19	28.5	27	29.6
20	28.7	28	29.7
21	28.8	29	29.8
22	28.9	30	30.0

have occurred in the whole system between the beginning and the end of the experiment, it is obviously preferable to take the second p_1 reading rather than the first.

Table I gives the osmotic pressure, expressed in mm. of toluene, developed by a 0.1 mm solution, in function of temperature. The calculations were based on the van't Hoff law and on the variation of the specific gravity of toluene with temperature (4).

The membrane being firmly held in place, no appreciable dilution can occur during the procedure. The amount of water which passes through the membrane until the final pressure is reached is exceedingly small. For example, a 0.1 mm solution will cause a rise of the toluene column of about 30 mm. The capillary being 0.2 mm. in inner diameter, the amount of liquid displaced will

be $0.1^2 \times 3.14 \times 30 = 0.94$ c.mm. The capacity of chamber h being about 200 c.mm., this will mean a dilution of less than 0.5 per cent. A correction can be calculated for the purpose. For the same reason, the increase in pressure will not affect appreciably either the height of fluid in h' , or the capillary forces at the water-toluene interface in q .

Since the fluid to be tested fills entirely the U formed by the lower arm of the tube, and chambers m and h being at the same level, no correction for specific gravity is necessary. No capillarity correction is necessary for obvious reasons.

Toluene has been chosen because, besides being almost non-miscible with water, it is remarkably fluid and never adheres in the capillary tube (water for that purpose would be quite unsuitable). It should be noted that when the apparatus is dismantled the manometer should not be emptied, but kept as it is in a vertical position with its lower end dipping in water. It can thus be used indefinitely.

The apparatus can be conveniently used also for the determination of higher pressures by connecting, through a section of rubber tubing, the toluene manometer with a water manometer. The procedure then simply consists, after closing the stop-cock, in keeping the toluene meniscus approximately immobile by increasing progressively the counterpressure in the water manometer until equilibrium is almost reached. The apparatus is then left alone while the toluene meniscus climbs a few mm. and finally remains immobile. The final reading is given by the sum of the toluene pressure difference ($p_2 - p_1$) and the water counterpressure applied. The specific gravity of toluene at room temperature is about 0.865 (5).

In order to test the apparatus, we prepared human hemoglobin solutions in the following way (6).

Oxalated blood was centrifuged, the serum removed, and the cells washed twice with 10 times their volume of 1.5 per cent NaCl. To 1 cc. of packed cells were added 0.3 cc. of water, 0.3 cc. of ether, and 0.2 gm. of solid NaCl. The solution obtained was centrifuged and filtered. It was then dialyzed against distilled water at room temperature in a little cellophane bag. The bag was held flat against a hard rubber frame, so as to offer the greatest possible surface in proportion to the volume of solution.

It was thus rocked in a trough for a few hours until the outer fluid, which was often renewed, remained salt-free. A mixture of Sørensen's phosphate solution was then added to the hemoglobin so as to make the final concentration $m/30$ and the pH 6.8. Under these conditions it is generally agreed (7) that the protein has a molecular weight of approximately 68,000. From this stock solution several more dilute solutions were prepared. They would keep in the ice box for weeks, without any apparent alteration. Spectroscopic examination showed no appreciable met-hemoglobin. As the proportion of total nitrogen, 16.75 per cent was used in the calculation (6). Total nitrogen determinations were made with the Van Slyke gasometric apparatus (8).

The difficulty of the method lies obviously in the use of a suitable membrane. Cellophane, of the kind which is quite satisfactory for dialysis, proved useless. With it, the pressure would rise a few mm., then stop abruptly and remain unchanged; no better result was ever obtained. With home-made collodion membranes the first results were not too unsatisfactory, but it was felt that a commercial product, if available, would give better guarantees of reproducibility. Therefore cellulose membranes manufactured according to Zsigmondy and listed as "ultrafine medium"¹ were tried and proved quite suitable.

Since these membranes are not very accurately graded, a few precautions are necessary. One large piece, as sold, can be cut into several small disks to fit the size of the apparatus. It is probably safe to test every one of these with a standard hemoglobin solution before using them further.

Since even membranes that seemed good at first sometimes later behaved abnormally, it was observed that plotting a time curve of the pressure change was helpful in eliminating erratic results, and that the two following conditions should be satisfied for the obtention of the most accurate figures.

1. The pressure-time curve should be quite smooth and regular, $\Delta p/\Delta t$ remaining always positive and decreasing slowly until equilibrium is reached.

2. Equilibrium should remain unchanged for at least 3 hours, with variations no greater than ± 0.1 mm.

¹ Sold by Pfaltz and Bauer, Inc., New York.

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Generally speaking, when a result is wrong, the final figure cannot be read exactly; it usually comes out 20 or 30 per cent

TABLE II
*Molecular Weight Determination of Hemoglobin; Concentration
0.186 Per Cent; Temperature 21°*

The values are given in mm.

Osmometer No.....	1	2	3	4
11.25 a.m.	16.3	11.2	8.0	5.9
1.10 p.m.	22.9	16.2	13.2	12.0
2.20 "	23.2	17.3	14.3	12.6
3.27 "	22.9	17.9	14.8	12.9
4.40 "	22.4	18.1	15.0	13.0
5.55 "	22.0	18.2	15.1	13.0
6.40 "	21.9	18.2	15.1	13.0
9.00 "	22.1	18.1	15.0	13.0
(Stop-cocks opened)				
9.20 p.m.	16.3	10.6	7.7	5.4
	22.1	18.2	15.1	13.0
	-16.3	-10.6	-7.7	-5.4
$p = p_2 - p_1 =$	5.8	7.6	7.4	7.6
Mol. wt. = $\frac{28.8 \text{ mm.}}{p} \times$ $0.186 \times 10^6 =$		71,000	72,000	71,000

TABLE III
*Molecular Weight of Human Hemoglobin in $\mu/30$ Sørensen's
Phosphate Buffer at pH 6.8*

Normal subject		Patient with nephrosis		
Concentration				
0.176 per cent	0.352 per cent	0.704 per cent	0.186 per cent	0.650 per cent
73,000	65,800	67,400	74,000	68,000
70,000	66,300	69,600	71,000	65,000
	70,000		73,000	67,100
	69,500		69,000	
	68,000		71,000	
			73,000	
			71,000	

too low, and its time curve is flagrantly abnormal, so that it can be excluded most easily. Table II gives an example.

Obviously the curve obtained from Osmometer 1 is unsatisfactory and should be rejected.

In the results given in Table III, all final figures read remained constant for at least 3 hours (sometimes much longer). As some of the determinations were allowed to stand overnight, complete time curves were not obtained for all, but there was no indication that important irregularities had occurred.

No particular claim is made as to the absolute accuracy of the molecular weights obtained, since they are reported primarily as an illustration of the method. The figures found for the smallest concentrations are by a few per cent above the molecular weight usually quoted. Whether this is due to a systematic experimental error (for which the membrane would most likely be responsible) or to the fact that at infinite dilution the correct figure is actually about 71,000 cannot be said yet. It should be borne in mind that the pressures read in that case are less than 8 mm. of toluene.

SUMMARY

An apparatus is described with which low osmotic pressures can be measured accurately on 0.2 cc. of solution and in less than 8 hours. Satisfactory results are obtained on solutions as little concentrated as 0.025 mm.

The method is illustrated with determinations of the molecular weight of human hemoglobin.

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THE SPECIFICITY OF PEPSIN

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New York)

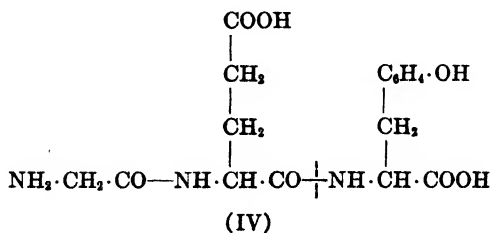
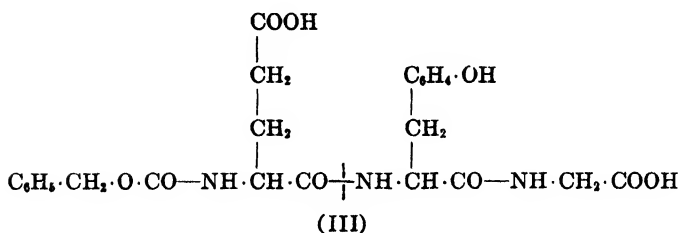
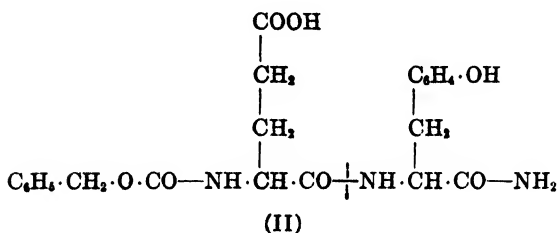
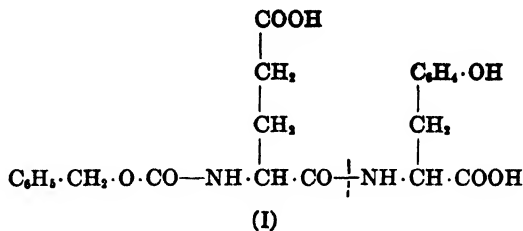
(Received for publication, December 13, 1938)

All the hitherto available information regarding the enzymatic action of pepsin has been obtained through the use of proteins as substrates. Much of this information must be regarded as tentative as long as no substrate of simple, well known structure can be provided for the study of pepsin action. It is the purpose of the present communication to report the finding of such substrates for swine pepsin.

The compound carbobenzoxy-*l*-glutamyl-*l*-tyrosine (I) is hydrolyzed to carbobenzoxyglutamic acid and tyrosine in the presence of crystalline swine pepsin. The hydrolysis occurs optimally at about pH 4 (Fig. 1). At pH 1.8 to 2, which is generally accepted as the optimum for pepsin, the hydrolysis of substrate (I) occurs rather slowly. Repeated recrystallization of the pepsin preparation did not appreciably alter its activity towards substrate (I). On the other hand, inactivation of pepsin at pH 8.0, followed by readjustment of the activity to pH 4.0, resulted in loss of the hydrolytic activity toward the substrate. The possibility that the splitting of substrate (I) by crystalline pepsin might be due to the presence of another enzyme of the cathepsin type is unlikely, since neither cysteine nor hydrogen peroxide had any effect on the rate of hydrolysis. Furthermore, there was no demonstrable carboxypeptidase activity in the enzyme preparations employed. The results of all these experiments justify the conclusion that the splitting of carbobenzoxyglutamyltyrosine is attributable to the action of pepsin itself (Table I).

The specificity of the pepsin action at pH 4 was investigated by means of other synthetic compounds differing from carbo-

benzoylglutamyltyrosine to a greater or lesser degree. Substitution of the tyrosine portion of the peptide by various amino acids showed that while carbobenzoxy-*l*-glutamyl-*l*-phenylalanine



was split fairly rapidly by pepsin with an optimum at about pH 4.5 (*cf.* Fig. 1), carbobenzoxy-*l*-glutamyl-*l*-diiodotyrosine, carbobenzoxy-*l*-glutamyl-*l*-glutamic acid, and carbobenzoxy-*l*-glutamyl-

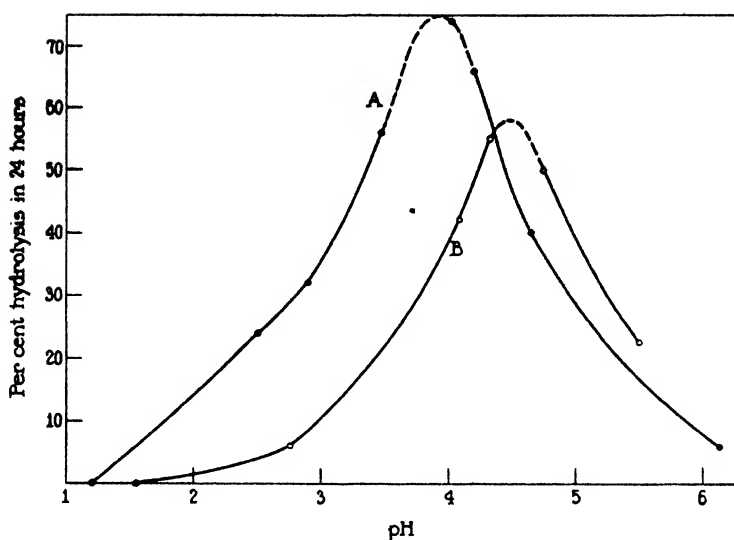


FIG. 1. pH dependence of hydrolysis of synthetic substrates by crystalline pepsin. Curve A, carbobenzoxy-*l*-glutamyl-*l*-tyrosine; Curve B, carbobenzoxy-*l*-glutamyl-*l*-phenylalanine. Enzyme concentration, 2.4 mg. of pepsin N per cc.

TABLE I

*Hydrolysis of Carbobenzoxyglutamyltyrosine with Crystalline Pepsin**

Enzyme preparation	Time	Hydrolysis
	hrs.	per cent
Twice crystallized pepsin, 1.4 mg. protein N per cc.	24	53
“ “ “ inactivated at pH 8 and readjusted to pH 4	24	0
4 times crystallized pepsin, 1.5 mg. protein N per cc.	24	54
Twice crystallized pepsin, 1.4 mg. protein N per cc.	22.5	51
“ “ “ + cysteine (0.005 mM per cc.)	46.5	72
“ “ “ + cysteine (0.005 mM per cc.)	22.5	53
“ “ “ + cysteine (0.005 mM per cc.)	46.5	72
Twice crystallized pepsin, 0.8 mg. protein N per cc.	24	33
“ “ “ + hydrogen peroxide (0.2 mM per cc.)	24	35

* pH 4.0.

glycine were resistant to pepsin action (Table II). On the other hand, substitution of the glutamic acid residue indicated that both carbobenzoxyglycyl-*l*-tyrosine and carbobenzoxy-*l*-tyrosyl-*l*-tyrosine were split by pepsin at a rather slow rate. All the peptides which were found in these experiments to be hydrolyzed by swine

TABLE II
*Behavior of Synthetic Substrates toward Crystalline Pepsin**

Substrate	Time		Isolation of products
	hrs.	Hydrolysis per cent	
Carbobenzoxy- <i>l</i> -glutamyl- <i>l</i> -tyrosine	6	18	Carbobenzoxy- <i>l</i> -glutamic acid
	24	64	
	48	81†	
Carbobenzoxy- <i>l</i> -glutamyl- <i>l</i> -phenylalanine	24	26	Tyrosine
	48	51	
	96	93	
Carbobenzoxy- <i>l</i> -glutamyl- <i>l</i> -diiodotyrosine	24	0	
	48	-1	
Carbobenzoxy- <i>l</i> -glutamyl- <i>l</i> -glutamic acid	24	3	
	48	3	
	96	5	
Carbobenzoxy- <i>l</i> -glutamylglycine	24	2	
	48	3	
	96	5	
Carbobenzoxy- <i>l</i> -tyrosyl- <i>l</i> -tyrosine	24	8	
	48	16	
	96	29	
Carbobenzoxyglycyl- <i>l</i> -tyrosine	24	11	
	48	30	
	96	43	

* pH 4.0; 1.6 mg. of pepsin N per cc.

† Beginning of tyrosine crystallization. If the reaction is allowed to proceed, about 50 per cent of the liberated tyrosine crystallizes out.

pepsin contain the aromatic amino acid residues tyrosine or phenylalanine. In the most sensitive substrates, tyrosine is combined with glutamic acid. On the basis of these experiments one may infer that the sensitivity of a peptide bond toward pepsin depends upon the nature of both amino acid residues which participate in the peptide bond that is hydrolyzed. However, there

are still other structural details of the substrate molecule which influence its sensitivity towards pepsin.

All the previously mentioned synthetic substrates for pepsin contain a free α -carboxyl and a free γ -carboxyl close to the peptide bond which is hydrolyzed by the enzyme. Substitution of the α -carboxyl of substrate (I) to yield carbobenzoxy-*l*-glutamyl-*l*-tyrosineamide (II) strongly depresses the rate of hydrolysis but

TABLE III
*Behavior of Synthetic Substrates toward Crystalline Pepsin**

Substrate	Time	Hy-	Isolation of products
		drol- ysis	
	<i>hrs.</i>	<i>per cent</i>	
Carbobenzoxy- <i>l</i> -glutamyl- <i>l</i> -tyrosine	24	53	Carbobenzoxy- <i>l</i> -glutamic acid Tyrosine
	48	74	
Carbobenzoxy- <i>l</i> -glutamyl- <i>l</i> -tyrosineamide	43	11	
	68	25	
Carbobenzoxy- <i>l</i> -glutaminyl- <i>l</i> -tyrosineamide	43	3	
	68	5	
Carbobenzoxy- <i>l</i> -glutamyl- <i>l</i> -tyrosylglycine	24	39	
	48	68	
Carbobenzoxyglycyl- <i>l</i> -glutamyl- <i>l</i> -tyrosine	24	48	
	48	52†	
<i>l</i> -Glutamyl- <i>l</i> -tyrosine	74	3	
Glycyl- <i>l</i> -glutamyl- <i>l</i> -tyrosine	24	32	
	48	43	
Benzoyl- <i>l</i> -tyrosine	48	2	
Chloroacetyl- <i>l</i> -tyrosine‡	24	0	

* pH 4.0; 1.4 mg. of pepsin N per cc.

† Tyrosine crystallization.

‡ An experiment at pH 7.0 also showed no hydrolysis of this substrate by the pepsin preparation.

does not eliminate it entirely. However, if both carboxyls are masked to give carbobenzoxy-*l*-glutaminyl-*l*-tyrosineamide, the product is entirely resistant to pepsin action (Table III). Furthermore, substitution of only the γ -carboxyl, as in carbobenzoxy-*l*-glutaminyl-*l*-phenylalanine, results in a slowing down of the hydrolysis, when compared with the hydrolysis of the acid containing two carboxyls (Table IV). One may conclude, therefore, that the presence of free carboxyls is favorable to the action

of pepsin if other structural requisites for the enzymatic action are fulfilled. In order to subject this conclusion to still another test, carbobenzoxy-*l*-glutamyl-*l*-tyrosylglycine (III) was investigated. The latter possesses a free α -carboxyl but at a greater distance from the glutamyltyrosine linkage than in (I). However, the rate of peptic hydrolysis of (III) is only slightly diminished when compared with that of (I); it is, however, remarkably increased when compared with that of the amide (II).

These results showing the favorable influence of free carboxyls lead to the expectation that pepsin action is inhibited by the presence, in the substrate, of a free amino group in close proximity

TABLE IV
*Behavior of Synthetic Substrates toward Crystalline Pepsin**

Substrate	Time	Hydrolysis
	<i>hrs.</i>	<i>per cent</i>
Carbobenzoxy- <i>l</i> -glutamyl- <i>l</i> -phenylalanine	24	24
	46	38
Carbobenzoxy- <i>l</i> -glutamyl- <i>d</i> -phenylalanine	24	5
	46	3
Carbobenzoxy- <i>l</i> -glutamyl- <i>l</i> -phenylalanine	20	8
	44	26
	92	50
Carbobenzoxy- <i>l</i> -phenylalanyl- <i>l</i> -glutamic acid	20	6
	44	20

* pH 4.2; 1.4 mg. of pepsin N per cc.

to the peptide bond. *l*-Glutamyl-*l*-tyrosine is completely resistant to the action of pepsin. Furthermore, the following compounds containing basic groups were tested for lability toward pepsin, with completely negative results at pH 2 and pH 4: benzoyl-*l*-lysineamide, benzoylglycyl-*l*-lysineamide, benzoyl-*l*-histidineamide, benzoylglycyl-*l*-histidineamide, benzoyl-*l*-arginineamide. However, the free tripeptide glycyl-*l*-glutamyl-*l*-tyrosine (IV) is split by pepsin with remarkable ease (Table III) between the glutamyl and tyrosine residues.

The specificity of pepsin is sensitive not only to the nature of the amino acids which participate in the peptide bond of the substrate, but also to the sequence of these amino acids. As an

example, carbobenzoxy-*L*-phenylalanyl-*L*-glutamic acid is hydrolyzed much more slowly than is carbobenzoxy-*L*-glutamyl-*L*-phenylalanine (Table IV).

The optical selectivity of pepsin is demonstrated in the resistance of carbobenzoxy-*L*-glutamyl-*D*-phenylalanine to the hydrolytic action of the enzyme (Table IV).

A few years ago Calvery and Schock (1) found that pepsin liberates tyrosine from egg albumin. It was rather difficult to reconcile this observation with the generally accepted opinion that all polypeptides were resistant to pepsin action. The hypothesis was discussed that, during the pepsin action on egg albumin, there might be formed peptides containing tyrosine in a special, hitherto unknown, and extremely labile combination (2). Hypotheses of this kind now become unnecessary, since it has been shown that pepsin splits peptide linkages of simple peptides, such as glycylglutamyltyrosine, with the liberation of tyrosine. The latter process is comparable to the formation of tyrosine during the pepsin action on egg albumin. Our experiments have demonstrated that pepsin is not restricted to the splitting of centrally located peptide bonds but also hydrolyzes terminal peptide bonds if they are situated at the carboxyl end of the peptide chain. The formation of tyrosine during the peptic digestion of a genuine protein probably proceeds in such a manner that at first the carboxyl group of tyrosine is liberated and that afterwards the peptide bond involving the amino group of tyrosine is hydrolyzed.

The enzymes that digest genuine proteins are usually classified as pepsinases, tryptases, or papainases on the basis of the pH optimum of their rather complex action on proteins (3). It is assumed that pepsin has its optimum around pH 1.8,¹ while the optimum of the papainases was found to depend upon the nature of the substrates and to vary from pH 3 to pH 10. However, the experiments with synthetic substrates have demonstrated the pepsin action to depend largely upon the structure of the substrate

¹ Dyckerhoff and Tewes (4) observed that the splitting of casein and gelatin by pepsin is, under certain conditions, more rapid at pH 4 than at pH 2. However, Northrop (5) has shown that the pH determinations performed by these authors by means of the indicator method were erroneous and that their experiments were actually carried out at about pH 2.35 and not at pH 4.

and to extend deeply into the range which was hitherto regarded as being reserved for the papainases. Consequently, it is not possible any longer to base the differentiation between pepsin and papainases on the assumption that pepsin has a single characteristic pH optimum. Another consequence is the following. If it is desired to investigate whether a given peptide or peptide derivative can be hydrolyzed by pepsin, a series of tests at different pH values must be performed. The splitting of carbobenzoxy-glutamylphenylalanine by pepsin would never have been observed if experiments had been performed only at pH 1.8.

The preference shown by pepsin to peptides containing glutamic acid² in addition to tyrosine or phenylalanine may partly explain the rapid and extensive hydrolysis by pepsin of proteins such as edestin, casein, and egg albumin (6) which contain many aminodicarboxylic acid residues as well as tyrosine and phenylalanine. The slow peptic hydrolysis of gelatin (6) corresponds to a very low content of aromatic amino acids. Protamines with a very high content of basic amino acids are either resistant to pepsin or attacked very slowly by the enzyme. This is in accord with the fact that no peptide derivative of well established structure and containing a basic amino acid as constituent is known to be digested by pepsin. Before a final conclusion is reached, however, it seems desirable to study a greater number of peptides which contain the residues of basic amino acids, and especially those peptides that contain the residues of basic amino acids as well as of aromatic amino acids.

Pepsin is not the only proteinase of the gastrointestinal tract that is specifically adapted to the hydrolysis of substrates containing tyrosine or phenylalanine. Chymotrypsin exhibits a similar preference towards the residues of these aromatic amino acids (7). Nevertheless, both enzymes exhibit distinctly different specificities. For example, pepsin hydrolyzes carbobenzoxy-glutamyltyrosine between the two amino acid residues, thus demonstrating that it is not inhibited by an α -carboxyl in close proximity to the peptide bond which is attacked. Chymotrypsin does not hydrolyze the previously mentioned substrate; the enzyme does, however, hydrolyze carbobenzoxytyrosylglycine-amide between the tyrosine and the glycine residues. Carbo-

² The behavior of peptides containing aspartic acid has not been investigated as yet.

benzoxytyrosylglycine is not attacked by chymotrypsin. Thus chymotrypsin is, in contrast to swine pepsin, highly sensitive towards the α -carboxyl. However, both enzymes are similar in that they do not require a basic group within their respective substrates. Therefore, the enzymatic hydrolysis of, for example, carbobenzoxyglutamyltyrosine by pepsin represents the interaction of an acidic enzyme and an acidic substrate.

The availability of synthetic substrates for pepsin having only one sensitive peptide bond permits a more precise study to be made of the kinetics of peptic hydrolysis and the quantitative estimation of pepsin in biological fluids. Furthermore, it permits a comparative study of the relative specificities of pepsins of various animal species to be undertaken.

The authors wish to express their thanks to Mr. S. Nagy who performed the analyses reported in this paper.

EXPERIMENTAL

*Carbobenzoxy-L-Glutamyl-L-Tyrosine*³—This compound was prepared as described in (8).

The enzymatic hydrolysate by pepsin of 554 mg. of this substance was filtered from a small amount of crystalline material which had separated during the reaction (15 mg. of tyrosine), concentrated to a small volume, and acidified to Congo red. The resulting oil was extracted with ethyl acetate. The ethyl acetate extract was dried and concentrated. Addition of petroleum ether resulted in the crystallization of a material (175 mg.) which after recrystallization from ethyl acetate-petroleum ether melted at 116°. When mixed with an authentic sample of carbobenzoxy-L-glutamic acid, the melting point was 118°. The aqueous layer from the ethyl acetate extraction was adjusted to pH 4 and cooled strongly. Tyrosine crystals separated out (32 mg.). Calculated for tyrosine, 7.7 per cent $\text{NH}_2\text{-N}$; found, 7.9 per cent $\text{NH}_2\text{-N}$.

Carbobenzoxy-L-Glutamyl-L-Phenylalanine

Carbobenzoxy-L-Glutamyl-L-Phenylalanine Ethyl Ester—To an ethyl acetate solution of phenylalanine ethyl ester (prepared

³ The glutamic acid residue in this compound and in the subsequent glutamyl peptides is linked to the next amino acid through the α -carboxyl group.

from 5 gm. of *l*-phenylalanine) 3.5 gm. of carbobenzoxyglutamic acid anhydride were added to a faintly alkaline reaction. The reaction mixture was allowed to stand for 3 hours, washed with dilute hydrochloric acid and water, and concentrated down. The resulting material was recrystallized from hot ethyl acetate. Yield, 4.2 gm. M.p., 144°.

$C_{24}H_{28}O_7N_2$.	Calculated.	C 63.1, H 6.1, N 6.1
456.5	Found.	" 62.8, " 5.9, " 6.1

Carbobenzoxy-l-Glutamyl-l-Phenylalanine—1.1 gm. of the above ester were shaken with 5 cc. of *N* NaOH for 15 minutes. On acidification to Congo red there separated a syrup which quickly crystallized. After recrystallization from ethyl alcohol-water the material melted at 162°. Yield, 0.8 gm.

$C_{22}H_{24}O_7N_2$.	Calculated.	C 61.7, H 5.6, N 6.5
428.4	Found.	" 61.6, " 5.7, " 6.6
$[\alpha]_D^{25} = +12.2^\circ$ (2.4% in <i>N</i> NaOH)		

Carbobenzoxy-l-Glutamyl-d-Phenylalanine

Carbobenzoxy-l-Glutamyl-d-Phenylalanine Ethyl Ester—This compound was prepared in the same manner as was the *l* form. M.p., 131°.

$C_{24}H_{28}O_7N_2$.	Calculated.	C 63.1, H 6.1, N 6.1
456.5	Found.	" 62.8, " 6.1, " 6.3

Carbobenzoxy-l-Glutamyl-d-Phenylalanine—This compound was prepared in the same manner as was the *l* form. M.p., 122°.

$C_{22}H_{24}O_7N_2$.	Calculated.	C 61.7, H 5.6, N 6.5
428.4	Found.	" 61.4, " 5.7, " 6.4
$[\alpha]_D^{25} = -21.5^\circ$ (2.4% in <i>N</i> NaOH)		

Carbobenzoxy-l-Glutamyl-l-Diiodotyrosine—1.7 gm. of carbobenzoxyglutamyltyrosine were dissolved in 25 cc. of concentrated ammonium hydroxide and 8 cc. of *N* I_2 -KI solution were added dropwise with shaking. On acidification to Congo red there resulted a gelatinous precipitate which was filtered off, washed with water, and dried. The pure diiodo compound (1.4 gm.) was obtained by crystallization from hot alcohol. M.p., 188°.

$C_{22}H_{22}O_4N_2I_2$.	Calculated.	C 37.9, H 3.2, N 4.0, I 36.5
	Found.	" 38.0, " 3.3, " 3.8, " 36.3

The same substance was also obtained by coupling carbo-benzoxyglutamic acid anhydride with diiodotyrosine methyl ester and saponifying the resulting ester.

Carbobenzoxy-L-Glutamyl-L-Tyrosineamide—1 gm. of the corresponding ester (1) was dissolved in 10 cc. of methyl alcohol saturated with ammonia at 0°. After 2 days at room temperature, the solution was evaporated down and the residue dissolved in dilute bicarbonate solution. The substance obtained on acidifying with dilute hydrochloric acid was recrystallized from dioxane-ether. M.p., 181°.

$C_{21}H_{21}O_7N_3$	Calculated.	C 59.6, H 5.7, N 9.5
443.4	Found.	" 59.4, " 5.8, " 9.4

Carbobenzoxy-L-Glutamyl-L-Tyrosineamide—2.1 gm. of carbo-benzoxyglutamyltyrosine ethyl ester were esterified in the usual manner with methyl alcohol saturated with hydrogen chloride. The syrupy ester obtained on evaporation was treated with a methyl alcohol solution of dry ammonia. The diamide crystallized out on allowing the reaction mixture to stand at room temperature. Yield, 1.8 gm. M.p., about 240°.

$C_{27}H_{26}O_8N_4$	Calculated.	C 59.7, H 5.9, N 12.7
442.5	Found.	" 59.4, " 6.1, " 12.6

Carbobenzoxy-L-Glutamyl-L-Tyrosylglycine

Carbobenzoxy-L-Glutamyl-L-Tyrosine Hydrazide—4.7 gm. of the corresponding ethyl ester (1) were treated with 1.5 cc. of hydrazine hydrate in 5 cc. of absolute alcohol. Slight warming gave a clear solution and on standing overnight the hydrazide separated out. Yield, 4.5 gm. On recrystallization from absolute alcohol the substance melted at 194°.

$C_{23}H_{26}O_7N_4$	Calculated.	C 57.6, H 5.7, N 12.2
458.5	Found.	" 57.4, " 5.6, " 11.9

Carbobenzoxy-L-Glutamyl-L-Tyrosylglycine Ethyl Ester—2.3 gm. of the above hydrazide were suspended in 25 cc. of ice-cold water, dissolved by the addition of 6 cc. of concentrated hydrochloric acid, and converted to the azide by the addition of 0.5 gm. of sodium nitrite. The azide was extracted with ethyl acetate and the extract was washed repeatedly with ice-cold water. The

dry azide solution was then added to a dry ether solution of glycine ethyl ester (from 5 gm. of the hydrochloride). After 24 hours the reaction mixture was washed with dilute hydrochloric acid and water. On evaporation of the ether-ethyl acetate layer, 1.7 gm. of the expected product were obtained. After recrystallization from dioxane-ether the melting point was 193–194°.

$C_{20}H_{31}O_9N_3$.	Calculated.	C 58.9, H 5.9, N 7.9
529.5	Found.	" 58.6, " 5.9, " 8.0

Carbobenzoxy-l-Glutamyl-l-Tyrosylglycine—2.5 gm. of the above ester were suspended in absolute alcohol and treated with 14 cc. of N NaOH. The resulting solution was left at 20° for 1 hour, acidified to Congo red with N hydrochloric acid, and concentrated down. The syrup which separated crystallized on standing. Yield, 2.0 gm. The material was air-dried for analysis. A sample dried *in vacuo* at 100° for 6 hours over P_2O_5 melted at 182°.

$C_{24}H_{37}O_9N_3 \cdot H_2O$.	Calculated.	C 55.5, H 5.6, N 8.1, H_2O 3.5
519.5	Found.	" 55.7, " 5.4, " 8.2, " 3.4

The enzymatic hydrolysate by papain of 250 mg. of this substance was concentrated to a small volume and acidified to Congo red. The resulting oil was extracted with ethyl acetate. The ethyl acetate layer was washed with water and then extracted with dilute bicarbonate solution. The bicarbonate extract was acidified to give a syrup which crystallized on standing in the ice chest. The dry material (86 mg.) melted at 118–120° and gave a mixed melting point of 119° with an authentic sample of carbobenzoxy-*l*-glutamic acid.

Carbobenzoxy-l-glutamic acid

$C_{12}H_{15}O_6N$.	Calculated.	C 55.5, H 5.4, N 5.0
281.2	Found.	" 55.3, " 5.3, " 5.0

Carbobenzoyglycyl-l-Glutamyl-l-Tyrosine

l-Glutamyl-l-Tyrosine Ethyl Ester—4.7 gm. of carbobenzoxy-*l*-glutamyl-*l*-tyrosine ethyl ester were hydrogenated in alcohol in the usual manner. Yield, 3.2 gm. The substance was recrystallized from hot alcohol. M.p., 144°.

$C_{16}H_{22}O_6N_2$.	Calculated.	C 56.7, H 6.6, N 8.3
338.4	Found.	" 56.4, " 6.4, " 8.2

Carbobenzoxyglycyl-L-Glutamyl-L-Tyrosine Diethyl Ester—3.1 gm. of glutamyltyrosine ester were esterified twice in the usual manner with ethanol-HCl. The syrupy diethyl ester hydrochloride was converted to the free ester with potassium carbonate. To the ethyl acetate solution of the free ester, 2.3 gm. of carbobenzoxyglycyl chloride were added in two portions with shaking. The reaction mixture was then shaken with a dilute bicarbonate solution. The ethyl acetate layer was then washed successively with water, dilute hydrochloric acid, and water. Crystallization occurred when the dried ethyl acetate solution was concentrated down. Yield, 4.3 gm. The substance was recrystallized from ethyl acetate. M.p., 169°.

$C_{25}H_{34}O_8N_2$.	Calculated.	C 60.3, H 6.3, N 7.5
557.6	Found.	" 60.3, " 6.4, " 7.6

Carbobenzoxyglycyl-L-Glutamyl-L-Tyrosine—2.8 gm. of the diethyl ester were suspended in 25 cc. of ethanol and with cooling there were slowly added 15 cc. of N NaOH. After 1 hour, 16 cc. of N HCl were added (Congo red acidity). The material which crystallized out on standing was filtered off and air-dried. Yield, 2.1 gm. A sample dried *in vacuo* at 100° for 5 hours over P_2O_5 melted at 173°.

$C_{24}H_{31}O_8N_2 \cdot H_2O$.	Calculated.	C 55.5, H 5.6, N 8.1, H_2O 3.5
519.5	Found.	" 55.3, " 5.6, " 8.0, " 3.7

During the enzymatic hydrolysis of 130 mg. of this substance by pepsin a crystalline precipitate separated out. It was filtered off and dried (20 mg.). Calculated for tyrosine, 7.7 per cent of NH_2-N ; found, 7.8 per cent of NH_2-N .

Glycyl-L-Glutamyl-L-Tyrosine—1 gm. of the carbobenzoxy compound was hydrogenated in the usual manner. The tripeptide crystallized out on evaporation of the filtrate. Yield, 0.6 gm.

$C_{16}H_{21}O_7N_3 \cdot 2\frac{1}{2}H_2O$.	Calculated.	C 46.6, H 6.4, N 10.2, H_2O 10.9
412.3	Found.	" 46.8, " 6.4, " 10.2, " 10.9

Carbobenzoxy-L-Glutaminyl-L-Phenylalanine

Carbobenzoxy-L-Glutaminyl-L-Phenylalanine Ethyl Ester—2.15 gm. of carbobenzoxyglutamylphenylalanine ethyl ester were suspended in 35 cc. of dry chloroform. 1.1 gm. of PCl_5 were added, followed

by shaking for 5 minutes at 0° and subsequent removal of the HCl by evacuation for 10 minutes. The solution was then added with cooling to an ethereal solution of ammonia. After the solution had stood at room temperature for 1 hour, the precipitate was filtered off, washed thoroughly with cold water, and dried. Yield, 1.5 gm. M.p., 138°.

$C_{24}H_{29}O_6N_3$.	Calculated.	C 63.3, H 6.4, N 9.2
455.5	Found.	" 63.4, " 6.4, " 9.1

Carbobenzoxyl-L-Glutaminyl-L-Phenylalanine—1.15 gm. of the above ester were dissolved in 60 cc. of alcohol and 2.5 cc. of N NaOH were added. The solution was acidified to Congo red after standing 1 hour at room temperature. The substance crystallized out upon concentration under reduced pressure. Yield, 0.9 gm. M.p., 180°.

$C_{22}H_{25}O_6N_3$.	Calculated.	C 61.8, H 5.9, N 9.8
427.4	Found.	" 61.9, " 6.1, " 9.7

Carbobenzoxyl-L-Phenylalanyl-L-Glutamic Acid

Carbobenzoxyl-L-Phenylalanyl-L-Glutamic Acid Diethyl Ester—3.5 gm. of carbobenzoxyl-L-phenylalanyl chloride were added to a dry ether solution of glutamic acid diethyl ester (prepared from 6 gm. of the hydrochloride). The mixture was allowed to stand at room temperature for 1 hour and washed with dilute hydrochloric acid, water, and bicarbonate solution. The dried ethereal solution was concentrated, yielding a crystalline product. Yield, 3.7 gm. M.p., 115°.

$C_{26}H_{32}O_7N_2$.	Calculated.	C 64.4, H 6.7, N 5.8
484.5	Found.	" 64.5, " 6.7, " 5.8

Carbobenzoxyl-L-Phenylalanyl-L-Glutamic Acid—2.4 gm. of the above ester were shaken with a mixture of 10 cc. of N NaOH and 10 cc. of alcohol. After 1 hour the solution was acidified to Congo red with N hydrochloric acid and concentrated under reduced pressure. The resulting crystals were filtered off and washed with cold water. Yield, 1.5 gm. M.p., 180°.

$C_{22}H_{24}O_7N_2$.	Calculated.	C 61.7, H 5.6, N 6.5
428.4	Found.	" 61.5, " 5.7, " 6.5

Carbobenzoxyl-L-Glutamyl-L-Glutamic Acid—This compound was prepared as described in (8).

Carbobenzoxyl-L-Tyrosyl-L-Tyrosine—This compound was prepared as described in (8).

l-Glutamyl-L-Tyrosine—This compound was prepared as described in (8).

Carbobenzoxyl-L-Glutamylglycine—This compound was prepared as described in (9).

Carbobenzoxylglycyl-L-Tyrosine—This compound was prepared as described in (7).

Enzymatic Studies

The crystalline pepsin was prepared according to the directions of Philpot (10). Unless otherwise stated, twice crystallized preparations were employed. Solutions of the enzyme were made up in acetate buffer of pH 4.0.

The concentration of the synthetic substrates was 0.05 mM per cc. in all cases. The solutions were buffered by 0.1 M acetate buffers. The temperature in all cases was 40°. The extent of hydrolysis was followed by the determination of the amino nitrogen liberated in the Van Slyke microvolumetric apparatus. Enzyme blanks and controls to test the lability of the substrates in the absence of pepsin were performed throughout these investigations.

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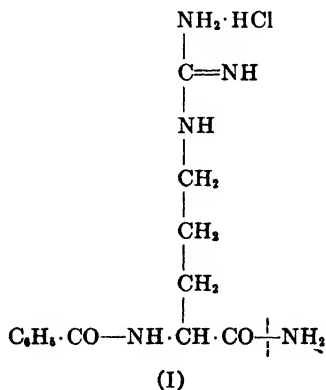
THE SPECIFICITY OF TRYPSIN

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(Received for publication, December 13, 1938)

The isolation of two proteinases in crystalline form from extracts of beef pancreas has been described by Kunitz and Northrop (1). These enzymes have been named trypsin and chymotrypsin. Previous publications (2) from this laboratory have reported the finding of a series of synthetic peptide derivatives which were readily hydrolyzed by crystalline chymotrypsin. In this communication a synthetic substrate for crystalline trypsin is described.

α -Benzoyl-L-arginineamide hydrochloride (I) is hydrolyzed extremely rapidly by crystalline trypsin (recrystallized three times) to yield benzoyl-L-arginine and ammonia (*cf.* Table I). This hydrolysis proceeds optimally at about pH 7.8 (Fig. 1).



Thus far, this compound is the only peptide-like derivative that has been found to act as a substrate for crystalline trypsin. Even

the closely related α -toluenesulfonyl-*l*-arginineamide hydrochloride is not hydrolyzed. Experiments with a series of acylamino acid

TABLE I
Action of Proteinases on Benzoyl-l-Arginineamide

Enzyme	pH	Time	Hydrolysis	Isolation of products
		hrs.	per cent	
Trypsin, 0.25 mg. protein N per cc.	7.8	1	50	Benzoyl- <i>l</i> -arginine
		2	64	
		3	80	
		20	95	
		43	95	
Chymotrypsin, 0.7 mg. protein N per cc.	7.8	2	1	
		19.5	3	
		43	4	
Papain-HCN*	5.0	2	76	
		6	96	
		24	99	
Bromelin-HCN*	5.0	6	45	
		24	77	

* The experiments with papain and bromelin were set up exactly as described in the experimental part of (3).

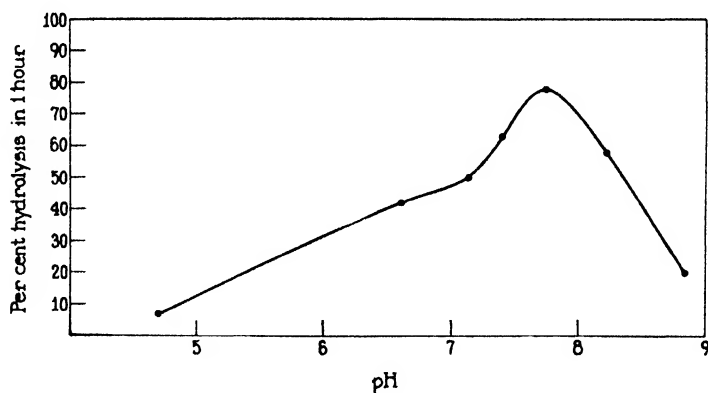


FIG. 1. pH dependence of hydrolysis of benzoyl-*l*-arginineamide by crystalline trypsin. Enzyme concentration 0.4 mg. of trypsin N per cc.

amides containing histidine, lysine, glutamic acid, tyrosine, and glycine gave negative results. Furthermore, trypsin is unable to

split the substrates of chymotrypsin (benzoyl-*L*-tyrosylglycine-amide) or heterotrypsin (α -hippuryllysineamide) (*cf.* Table II).

The differences in chemical specificity exhibited by the three pancreatic enzymes trypsin, chymotrypsin, and heterotrypsin permit of a determination of their respective activities in crude pancreatic extracts. Such a study is at present in progress in this laboratory. The availability of the synthetic substrates also facilitates the study of the relationship of the pancreatic proteinases to enzymes obtained from other biological sources and named "trypsins" because of their optimal action on proteins in an alkaline milieu.

TABLE II
*Behavior of Synthetic Substrates in Presence of Trypsin**

Substrate	Time	Hydrolysis
	<i>hrs.</i>	<i>per cent</i>
Toluenesulfonyl- <i>L</i> -arginineamide	24	-1
Benzoyl- <i>L</i> -histidineamide	42	3
Benzoyl- <i>L</i> -lysineamide	42	2
Carbobenzoxy- <i>L</i> -isoglutamine	48	3
Benzoyl- <i>L</i> -tyrosineamide	48	0
Hippurylamide	48	1
Benzoyl- <i>L</i> -tyrosylglycineamide	24	0
α -Hippuryl- <i>L</i> -lysineamide ..	24	4

* pH 7.8; 0.4 mg. of trypsin N per cc.

The behavior of benzoylarginineamide when subjected to the action of other proteinases is of some interest (*cf.* Table I). The resistance of this compound to chymotrypsin emphasizes the difference in chemical specificity of trypsin and chymotrypsin. Furthermore, the rapid hydrolysis of this substrate by the intracellular enzymes papain and bromelin is noteworthy. The use of benzoylarginineamide for the study of the activation of papain has been described elsewhere (4).

The closer study of the specificity of crystalline trypsin, with suitably substituted peptides of arginine and other amino acids, is contemplated.

EXPERIMENTAL

 α -Benzoyl-L-Arginineamide Hydrochloride¹

Benzoyl-L-Arginine—To a solution of 21.2 gm. of arginine monohydrochloride in 120 cc. of H_2O there were added in several portions 12.8 cc. of benzoyl chloride and a solution of 17.2 gm. of sodium carbonate in 170 cc. of water. The reaction mixture was stirred vigorously and the temperature kept at about 20° . The addition of the reagents required $2\frac{1}{2}$ hours, and after another $1\frac{1}{2}$ hours of stirring the reaction mixture was acidified to Congo red and the slight precipitate (dibenzoylarginine) filtered off. The filtrate was extracted with ether and neutralized to about pH 8 with ammonia. Upon concentration under diminished pressure, crystallization occurred. Yield, 20 gm. The material was recrystallized from hot water.

$C_{13}H_{18}O_4N_4$	Calculated.	C 56.0, H 6.5, N 20.2
278.3	Found.	" 55.8, " 6.4, " 19.9

Benzoyl-L-Arginineamide Hydrochloride—7.6 gm. of the above compound were esterified in the usual manner (with cooling) in 350 cc. of absolute methanol. After the reaction mixture had stood in the ice box overnight, the solution was concentrated at 30° . The residue was dissolved in a small volume of methanol and the syrupy ester precipitated by means of dry ether. This material was reesterified and the resulting syrup was dissolved in 70 cc. of methanol previously saturated with dry ammonia at 0° . After it had stood 2 days at room temperature, the solution was concentrated and the resulting syrup taken up in hot water. The material which crystallized on cooling sintered at 120 – 123° , lost water of crystallization at 135 – 140° , and decomposed above 260° . Yield, 3.7 gm.

$C_{13}H_{20}O_2N_4Cl \cdot H_2O$	Calculated.	C 47.1, H 6.6, N 21.1
331.8	Found.	" 47.0, " 6.6, " 21.0

The enzymatic hydrolysate by trypsin of 498 mg. of this compound was filtered, concentrated to a small volume, and placed

¹ This compound has already been synthesized by Dirr and Späth (5). In view of its value as a substrate for trypsin, a fuller description of the synthesis is presented.

in the ice box. The resulting crystals were collected, washed with cold water, dried (288 mg.), and recrystallized from hot water.

Benzoyl-L-arginine

$C_{13}H_{15}O_3N_4$	Calculated.	C 56.0, H 6.5, N 20.2
278.3	Found.	" 55.9, " 6.5, " 19.8

During the enzymatic hydrolysis the liberation of ammonia was also observed.

α -Toluenesulfonyl-L-Arginineamide

α -Toluenesulfonyl-L-Arginine—This compound has already been described as an oil by McChesney and Swann (6). It was obtained in crystalline form by the following procedure.

21 gm. of arginine monohydrochloride were dissolved in 115 cc. of 2 N NaOH and stirred with a solution of *p*-toluenesulfochloride in 400 cc. of ether. After about 25 minutes the formation of crystals began. 3 hours later the mixture was acidified by addition of acetic acid, and the crystals of toluenesulfonylarginine were filtered after several hours. Yield, 36 gm. On recrystallization from hot water, the substance melted at 256–257° (decomposition).

$C_{13}H_{19}O_4N_4S \cdot 3H_2O$	Calculated.	C 40.8, H 6.8, N 14.7, H_2O 14.1
382.3	Found.	" 40.7, " 6.7, " 14.6, " 14.1

α -Toluenesulfonyl-L-Arginineamide Hydrochloride—7 gm. of dry toluenesulfonylarginine were treated at 0° with 500 cc. of absolute methanol saturated with dry HCl. After 18 hours the methanol was evaporated off and the resulting syrup crystallized by means of ether. This material was treated with 60 cc. of absolute methanol previously saturated with dry ammonia at 0°. After 48 hours, the evaporated solution yielded crystals which were transferred to the filter by means of ether. Yield, 6.2 gm.

$C_{13}H_{17}O_2N_4SCl \cdot H_2O$	Calculated.	C 40.9, H 6.3, N 18.3, H_2O 4.7
381.8	Found.	" 41.0, " 6.2, " 18.4, " 4.8

Benzoyl-L-Histidineamide—1 gm. of monobenzoylhistidine methyl ester (7) was dissolved in 10 cc. of methanol previously saturated with dry ammonia. The amide crystallized on standing

at room temperature for 18 hours. After recrystallization from methanol the substance melted at 234°.

$C_{12}H_{14}O_2N_4$.	Calculated.	C 60.4, H 5.3, N 21.6
258.2	Found.	" 60.4, " 5.4, " 21.6

Benzoyl-L-Lysineamide—This substance was prepared as described in (8).

α -Hippuryl-L-Lysineamide—This substance was prepared as described in (8).

Carbobenzoxy-L-Isoglutamine—This substance was prepared as described in (9).

Benzoyl-L-Tyrosineamide—This substance was prepared as described in (2).

Benzoyl-L-Tyrosylglycineamide—This substance was prepared as described in (2).

Enzymatic Studies

Trypsin, recrystallized three times, and chymotrypsin (prepared as described in (1)) were employed. The concentration of the synthetic substrates was 0.05 mm per cc. in all cases. The solutions were buffered at pH 7.8 by means of m/15 phosphate buffers and at pH 5 by means of 0.04 M citrate buffers. The temperature was 40° in all experiments. The extent of hydrolysis was followed by microtitration of the liberated carboxyl groups according to the method of Grassmann and Heyde (10). Enzyme blanks and controls to test the lability of the substrates in the absence of the enzyme were performed.

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THE IDENTIFICATION OF EQUOL AS 7-HYDROXY-3-(4'-HYDROXYPHENYL) CHROMAN, AND THE SYNTHESIS OF RACEMIC EQUOL METHYL ETHER

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(Received for publication, December 9, 1938)

Equol, an optically active dihydroxyphenol of the formula $C_{16}H_{14}O_3$, was first isolated from the urine of pregnant mares by Marrian and Haslewood (1). Later Marrian and Beall (2) found that it could also be isolated from the urines of non-pregnant mares and of stallions, and they showed that on fusion with potassium hydroxide it yielded resorcinol, β -resorcylic acid, *p*-ethylphenol, *p*-hydroxybenzoic acid, and a phenol (B), not identical with equol itself, which had the approximate composition $C_{15}H_{14-16}O_3$ and which they believed was a trihydroxy compound. On the basis of the formation of these decomposition products it was suggested that equol must be 7-hydroxy-2-(4'-hydroxyphenyl) chroman (I), 7-hydroxy-3-(4'-hydroxyphenyl) chroman (II), or 6-hydroxy-2-(4'-hydroxybenzyl) coumaran (III).

More recently the constitution of equol has been investigated more fully by Wessely *et al.* (3). These workers have satisfactorily shown that the phenol (B) isolated by Marrian and Beall from the products of the fusion of equol with potassium hydroxide is a dihydroxy compound and not a trihydroxy one, and that it is not a primary product of decomposition but is an artifact produced by ring closure during distillation from the primary product (IV), the structure of which was proved by synthesis. Wessely *et al.*, therefore, believe that the phenol (B) is 6-hydroxy-2-(4'-hydroxyphenyl)-2-methyl coumaran (V) on the assumption that ring closure of (IV) during distillation occurs normally. The identification of the decomposition product (IV) enabled Wessely *et al.*

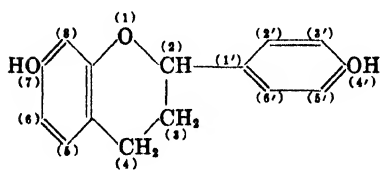
to narrow down the number of possible structures for equol to two, 7-hydroxy-3-(4'-hydroxyphenyl) chroman (II) and 6-hydroxy-2-(4'-hydroxyphenyl)-2-methyl coumaran (V), and they pointed out that if the latter formula were correct then phenol (B) would be the racemic form of equol itself.

Before the paper by Wessely *et al.* was published, a further investigation of the decomposition products of equol was in progress in this laboratory, and attempts were being made to synthesize compounds of the three structures suggested as possibilities by Marrian and Beall. It was found that equol methyl ether, $[\alpha]_{5461}^{23} = -19.5^\circ$, on oxidation with cold chromic anhydride yielded a compound $C_{17}H_{16}O_4$, which melted at $120.5-121.5^\circ$ and had $[\alpha]_{5461}^{25} = -88.7^\circ$. It seemed probable that this compound had been formed by the oxidation of a methylene group to a ketone group. On reduction of this compound by the Clemmensen method, a product melting at $112.5-114^\circ$ was obtained, which on analysis corresponds with the formula $C_{17}H_{18}O_3$; it was optically inactive. Since the melting point of equol methyl ether ($89.5-90.5^\circ$) was not depressed by admixture with this product, it was considered probable that it might be the racemic form of the former. In order to prove that racemization could occur under the conditions of the Clemmensen reduction, the ketone $C_{17}H_{16}O_4$ was heated with an acetic acid-hydrochloric acid mixture. The product, which melted at $126-126.5^\circ$, gave on analysis $C_{17}H_{16}O_4$ and was optically inactive. The same racemic product was also obtained by heating the optically active ketone in 70 per cent ethanol in the presence of sodium carbonate.

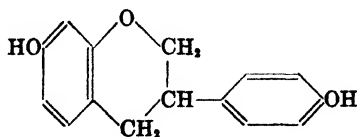
Before it was known by the present authors that equol could not be identical with 7-hydroxy-2-(4'-hydroxyphenyl) chroman (I), the dimethyl ether of this compound had been prepared synthetically by reduction of 7,4'-dimethoxyflavylium chloride with sodium amalgam. Since the product was found to depress the melting point of equol methyl ether, attempts to synthesize the dimethyl ether of 7-hydroxy-3-(4'-hydroxyphenyl) chroman (II) were commenced.

2-Hydroxy-4-methoxyphenyl-*p*-methoxybenzyl ketone (VI) was prepared by condensing *p*-methoxyphenylacetonitrile with resorcinol monomethyl ether according to the method of Baker and Robinson (4). By the interaction of (VI) with ethyl formate in

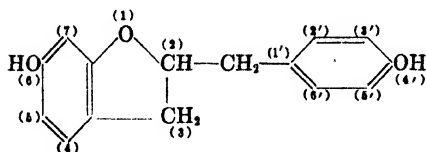
the presence of sodium, according to the method of Mahal, Rai, and Venkataraman (5), 7,4'-dimethoxyisoflavone (VII) was obtained. Attempts to reduce this isoflavone to the 2,3-dihydro



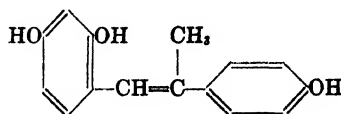
I



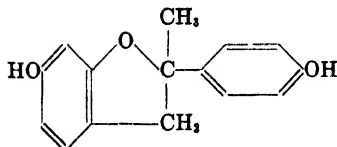
II



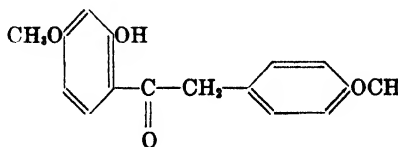
III



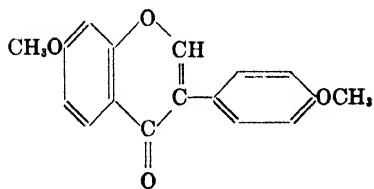
IV



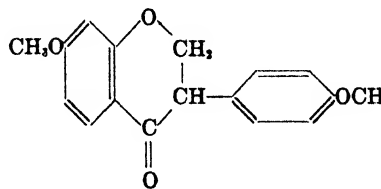
V



VI



VII



VIII

derivative by means of sodium amalgam were entirely unsuccessful. Catalytic reduction was therefore resorted to. The isoflavone was completely resistant to hydrogenation in neutral ethanolic solution with platinum oxide. When, however, glacial

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acetic acid was used as the solvent, hydrogenation proceeded rapidly; and by stopping the process after the uptake of just over 1 mole of hydrogen, good yields of a compound melting at 125.5–126° which on analysis gave $C_{17}H_{18}O_4$ were obtained. It was evident that the product was the desired 7,4'-dimethoxy-2,3-dihydroisoflavone (VIII), since it proved to be stable to oxidation with chromic anhydride and could readily be reduced to a compound of the formula $C_{17}H_{18}O_3$ by the Clemmensen method. The synthetic 7,4'-dimethoxy-2,3-dihydroisoflavone proved to be identical with the racemized oxidation product of equol methyl ether. Thus it was shown that equol must be 7-hydroxy-3-(4'-hydroxyphenyl) chroman (II). Confirmation of this conclusion was obtained when it was found that the Clemmensen reduction product of the synthetic dihydroisoflavone was identical with the Clemmensen reduction product of the oxidation product of equol methyl ether. By oxidation of the Clemmensen reduction product of the synthetic dihydroisoflavone with chromic anhydride, the compound (VIII) was regenerated. Thus it was clear that the reduction had followed a normal course and that therefore the former compound was indeed identical with racemic equol methyl ether itself.

EXPERIMENTAL

Oxidation of Equol Methyl Ether with Chromic Anhydride—To 620 mg. of equol methyl ether (m.p. 89.5–90.5°; $[\alpha]_{D}^{25} = -19.5^\circ$) dissolved in 20 cc. of 90 per cent acetic acid were added 620 mg. of chromic anhydride dissolved in 20 cc. of 90 per cent acetic acid. The mixture was allowed to stand at room temperature for 16 hours. The heavy white precipitate which separated after dilution of the oxidation mixture was filtered off and washed thoroughly with water. After being boiled with charcoal in ethanolic solution and crystallized twice from methanol, a white crystalline product, m.p. 120.5–121.5° (sintering at 119°), was obtained in a yield of 205 mg.

$C_{17}H_{18}O_4$.	Calculated.	C 71.80,	H 5.68
	Found.	" 71.78, 71.88,	" 5.67, 5.64
$[\alpha]_{D}^{25} = -88.7^\circ$ (3.4% in chloroform)			

Reduction of Oxidation Product by Clemmensen's Method—200 mg. of the oxidation product of equol methyl ether were dissolved in a mixture of 10 cc. of glacial acetic acid and 7.5 cc. of concentrated hydrochloric acid. 2 gm. of freshly prepared zinc amalgam were added and the mixture was then heated on a boiling water bath under a reflux condenser. After 3 hours, additional quantities of 2 gm. of zinc amalgam and 5 cc. each of acetic acid and hydrochloric acid were added, and the heating continued for a further 3 hours. When the reaction mixture was diluted heavily with water, a white flocculent precipitate separated out. This was filtered off and, after washing, was dissolved in ether. The ethereal solution was then washed repeatedly with water and evaporated to dryness. The residue, after treatment with charcoal in methanolic solution, was crystallized twice from that solvent. White crystals, m.p. 112.5–114° (sintering at 110°), were obtained in a yield of 59 mg. After admixture with equol methyl ether, the melting point was 90–111°.

$C_{17}H_{18}O_4$. Calculated. C 75.55, H 6.66
 Found. " 75.58, 75.32, " 6.76, 6.65
 $[\alpha]_{D}^{25} = 0^\circ$ (3.14 and 1.92% in chloroform)

Racemization of Oxidation Product. (a) *With Acid*—66 mg. of the oxidation product were heated at 100° for 6 hours with 10 cc. of a mixture of equal parts of glacial acetic and hydrochloric acids. The solution was diluted with 5 volumes of water and the precipitate that formed was filtered off, washed thoroughly with water, and crystallized once from aqueous ethanol and once from absolute methanol. 30 mg. of white crystals, melting at 126–126.5° (sintering at 125°), were obtained. After admixture with the untreated oxidation product, the melting point of the latter was not depressed.

$C_{17}H_{18}O_4$. Calculated. C 71.80, H 5.68
 Found. " 71.96, 71.71, " 5.72, 5.71
 $[\alpha]_{D}^{25} = 0^\circ$ (1.86% in chloroform)

(b) *With Alkali*—75 mg. of the oxidation product were dissolved in 40 cc. of 70 per cent ethanol to which were added 100 mg. of sodium carbonate. After the mixture was boiled under a reflux

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condenser for 30 minutes, the ethanol was evaporated under reduced pressure and the residue was thoroughly leached with water. The insoluble material, after being crystallized once from aqueous ethanol, yielded 62 mg. of white crystals melting at 126.5–127.5° (sintering at 125°).

$C_{17}H_{16}O_4$. Calculated. C 71.80, H 5.68
 Found. " 71.88, 71.86, " 5.68, 5.66
 $[\alpha]_{D}^{25} = 0^\circ$ (2.06% in chloroform)

2-Hydroxy-4-Methoxyphenyl-p-Methoxybenzyl Ketone—5.8 cc. of *p*-methoxyphenylacetonitrile and 5.8 cc. of resorcinol monomethyl ether were dissolved in 30 cc. of anhydrous ether. To the solution were added 1.5 gm. of freshly fused and powdered zinc chloride, and a stream of dry hydrogen chloride was then passed into the mixture for 2 hours.

After standing at 0° in a stoppered vessel for 48 hours, the ketimines were precipitated as a light brown gummy mass by the addition of 130 cc. of dry ether. This was separated and hydrolyzed by boiling for 1½ hours with 200 cc. of *N* hydrochloric acid. The ketones were separated from the hydrolysis mixture by extraction with ether. In order to remove the unwanted 2-methoxy-4-hydroxyphenyl-*p*-methoxybenzyl ketone which was formed in the reaction, the ethereal extract was washed with 1 per cent sodium hydroxide before being washed with water and evaporated to dryness. Crystallization of the residue yielded 1.95 gm. of a white product. For analysis, a small sample of this was further purified by crystallization twice from absolute methanol. The product melted at 102.5–104°.

$C_{18}H_{16}O_4$. Calculated. C 70.52, H 5.93
 Found. " 70.25, 70.04, " 5.95, 5.96

7,4'-Dimethoxyisoflavone—A solution of 1.2 gm. of 2-hydroxy-4-methoxyphenyl-*p*-methoxybenzyl ketone in 37.5 cc. of anhydrous ethyl formate was added to 1.2 gm. of sodium dust cooled in a freezing mixture over a period of 50 minutes with continuous stirring. After standing for 48 hours in a freezing mixture, ice was added and the mixture was extracted with ether. Evaporation of the ethereal solution yielded a brown oil which was dissolved in a small volume of ethanol. After standing for several

days the solution deposited crystals in a yield of 0.93 gm., which, after two recrystallizations from acetone, melted at 158–159°.

$C_{17}H_{14}O_4$.	Calculated.	C 72.31,	H 5.00
	Found.	" 72.38, 72.41,	" 5.00, 5.02

7,4'-Dimethoxy-2,3-dihydroisoflavone—A solution of 1.0 gm. of 7,4'-dimethoxyisoflavone in 200 cc. of glacial acetic acid, to which were added 40 mg. of platinum oxide, was shaken in an atmosphere of hydrogen. After 12 minutes, when 100 cc. of hydrogen had been absorbed (1.25 moles), the shaking was discontinued and the platinum removed from the solution by filtration. Dilution of the filtrate with 1000 cc. of water yielded a white crystalline precipitate which was filtered off and washed with water. After crystallization once from 95 per cent ethanol and once from 50 per cent acetone, a product melting at 122–123.5° was obtained in a yield of 0.7 gm. After two further crystallizations from absolute methanol, white prisms melting at 125.5–126° were obtained.

$C_{17}H_{14}O_4$.	Calculated.	C 71.80,	H 5.68
	Found.	" 71.76, 71.94,	" 5.70, 5.60

After treatment with chromic anhydride in the manner described for the oxidation of equol methyl ether, the original product was obtained unchanged. The mixed melting points, after admixture of the synthetic product with (a) the optically active oxidation product of equol methyl ether, (b) the acid-racemized oxidation product, and (c) the alkali-racemized oxidation product, were 119–125°, 125.5–126°, and 126–127° respectively.

7-Methoxy-3-(4'-Methoxyphenyl) Chroman—200 mg. of the synthetic 7,4'-dimethoxy-2,3-dihydroisoflavone were reduced by the Clemmensen method, as described previously. After three crystallizations from methanol, 42 mg. of white crystals, m.p. 112.5–114° (sintering at 111°), were obtained.

$C_{17}H_{18}O_2$.	Calculated.	C 75.55,	H 6.66
	Found.	" 75.44, 75.57,	" 6.77, 6.70

After admixture of the product with the compound obtained by Clemmensen reduction of the oxidation product of equol methyl

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ether, the melting point was unchanged. The mixed melting point with equol methyl ether was 89.5–108°.

Oxidation of 7-Methoxy-3-(4'-Methoxyphenyl) Chroman with Chromic Anhydride—25 mg. of synthetic 7-methoxy-3-(4'-methoxyphenyl) chroman were oxidized with chromic anhydride in 90 per cent acetic acid in the manner described for the oxidation of equol methyl ether. The melting point of the product was 126–127.5° (sintering at 125°) and was not depressed by admixture with 7,4'-dimethoxy-2,3-dihydroisoflavone.

$C_{17}H_{16}O_4$. Calculated, C 71.80, H 5.68; found, C 72.11, H 5.69

The authors wish to express their gratitude to Miss Dorothy Skill who carried out the microanalyses reported in this paper. They are also grateful to Dr. R. D. H. Heard of the Connaught Laboratories who generously supplied them with crude equol.

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THE DISTRIBUTION OF ELECTROLYTES IN MAMMALIAN TISSUES

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Although the determination of the inorganic composition of tissues dates back to the early nineteenth century, there has been renewed interest in this subject in recent years arising out of the desire to relate the morphologically separate phases of tissues to their chemical composition. The questions at issue are: (1) Can tissues be viewed as consisting of separate phases which are both morphologically and chemically distinct? (2) What are the relative proportions of these phases in tissues? (3) What are the compositions of the different phases? The present communication is an attempt to contribute evidence which will be of help in answering these questions.

Tissues can be analyzed as a whole, but, unfortunately, with the exception of blood, they cannot be separated for direct analysis into their individual phases. By inferences drawn from chemical analyses (Fenn (7); Eggleton *et al.* (6); Hastings and Eichelberger (12)) and by the direct application of histochemical methods (Gersh (9)), the following conclusions have been reached regarding living skeletal muscle. Muscle cells (*i.e.*, the intracellular phase of the tissue) contain a high concentration of potassium and phosphate, little if any sodium, and no chloride. The remainder, called the extracellular phase, on the other hand, approximates a serum ultrafiltrate in composition and contains all of the chloride and most of the sodium. Recent work by Manery, Danielson, and Hastings (19) indicates that the extra-

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cellular phase of muscle may more accurately be described as consisting of extracellular fluid and the connective tissue proteins. The observations of Harrison, Darrow, and Yannet (11) suggested that all living tissues may be divided into intra- and extracellular phases in the same manner as skeletal muscle. This hypothesis has been thoroughly discussed by Peters (20). By repeated perfusion of cats with a solution in which sulfate was substituted for chloride, Amberson *et al.* (1) demonstrated that, as the plasma chloride was reduced, the chloride of all tissues was reduced. In the case of muscle and many other tissues, the reduction in tissue chloride paralleled the reduction in plasma chloride. However, nervous tissues, stomach, and tendon were exceptions.

From the analyses to be presented in the present paper, we have been led to the conclusions (1) that the electrolytes are essentially identical in the extracellular fluid of all tissues, (2) that the mass of the extracellular phase, containing the extracellular fluid, varies from one type of tissue to another, and (3) that tissues have varying proportions of at least two types of cells: those which contain no sodium or chloride, and those which contain sodium and chloride.

Such a simplified and fragmentary description of the morphologically complex and functionally diverse tissues studied can obviously serve only as a first approximation. Its justification rests on the degree to which it provides a fruitful means of approach to further work.

Calculation

If all of the tissue chloride found by analysis were confined to the extracellular phase, as defined above for muscle, and if this phase were identical with an ultrafiltrate of serum, then, the chloride content of the tissue and of serum being known, the mass of the extracellular phase could be calculated. (In such a calculation the mass occupied by the connective tissue proteins is neglected.) If the water content of this phase is subtracted from the total tissue water, the quantity of intracellular water may be estimated. In the calculation which follows a Gibbs-Donnan equilibrium ratio between mammalian serum and its ultrafiltrate of 0.95 for diffusible univalent ions is used (Hastings *et al.* (13)). In these calculations, the water content of the extracellular phase has been assumed to be 99 per cent. The implications in this

assumption will be discussed subsequently. The following calculations have been applied to analytical data after corrections have been made for blood and fat contained in the tissue analyzed. All the original data are presented in Tables I, II, and III.

TABLE I
K, Cl, and H₂O of Tissues

	Tissue	H ₂ O per kilo wet weight	Cl per kilo tissue H ₂ O	K* per kilo tissue H ₂ O
		gm.	m.eq.	m.eq.
Rat (5)	Gastrocnemius	761	21.2	148.2
	Heart	780	31.3	107.8
	Brain	782	39.7	131.1
	Liver	721	41.7	127.0
	Spleen	766	45.1	148.0
	Testes	867	72.3	106.1
	Kidney	762	62.5	93.4
	Lung	797	71.0	107.2
	Serum	919	115.0	5.0
Human	Uterus (1)	805	59.7	74.3
Rabbit	Gastrocnemius (1)	753	19.4	152.8
	Ovaries (3)	724	48.9	81.3
	Fundic mucosa (1)	791	96.3	87.0
	Skin (1)	675	111.0	29.8
	Ear cartilage (1)	593	118.4	33.6
	Tendon (1)	596	123.5	16.5

The figures in parentheses represent the number of animals. The maximum deviation from the average in the ratio Cl:K for a single tissue of the five rats was 5 per cent.

* We are indebted to Miss Doris M. Cobb for the potassium analyses on rat tissues.

The method of calculation follows:

$$[\text{Cl}]_E = \frac{[\text{Cl}]_S}{0.95}$$

$$\frac{(\text{Cl})_T}{[\text{Cl}]_E} \times 1000 = (\text{H}_2\text{O})_E \text{ gm. per kilo tissue}$$

$$(\text{H}_2\text{O})_T - (\text{H}_2\text{O})_E = (\text{H}_2\text{O})_C \text{ gm. per kilo tissue}$$

$$(\text{H}_2\text{O})_E - 0.99 = (E) \text{ gm. per kilo tissue}$$

$$1000 - (E) = (C) \text{ gm. per kilo tissue}$$

$$\frac{1000}{(C)} \times (\text{H}_2\text{O})_C = (\text{H}_2\text{O})_C \text{ gm. per kilo cells}$$

TABLE II
Analyses of Rat Tissues

Rat No.	Chloride			Water		Blood			Fat			Chloride			Water			
	Per kilo fresh tissue						Per kilo blood and fat-free tissue											
	m. eq.	m. eq.	m. eq.	gm.	gm.	gm.	cc.	cc.	gm.	gm.	gm.	m. eq.	m. eq.	m. eq.	gm.	gm.	gm.	
6	7	8	6	7	8	6	7	8	6	7	8	6	7	8	6	7	8	
Skin.....	63.8	58.3	61.0	634	618	604	0	0	14.6	41.9	86.9	64.8	60.8	66.8	643	645	660	
Subcutaneous tissue.....	63.6	53.1	51.3	723	636	497	0	0	57.3	204.0	420.0	67.5	66.6	88.4	768	798	857	
Testes.....	65.1	63.1	62.9	873	870	871	0.6	1.0	11.1	9.8		66.0	63.7	63.0	883	879	871	
Liver.....	32.9	25.3	31.0	702	713	706	34.0	19.0	26.1	18.7	7.6	6.3	31.5	24.2	28.7	714	707	
Kidney.....	54.4	58.1		764	767	773	54.0	62.0	47.1	16.9	8.8	6.7	53.4	56.5	778	775	777	
Spleen*.....	41.6	39.2	45.2	775	771	779	171.0	152.0	163.0	12.6	8.4	36.6	34.3	41.3	812	800	814	
Lung.....	65.1	57.2		793	791	853	185.0	190.0	153.0	21.5	24.1	8.6	61.6	51.3	823	835	873	
Stomach.....	59.8	62.0	67.1	748	743	719	6.2	5.5	11.7	42.0	26.5	41.8	62.1	64.2	69.8	781	770	
Small intestine.....		47.4	50.6		695	649		4.1			49.6		49.6	53.0		730	720	
Large ".....	34.8		42.3	791		752	4.8		47.3			38.7	36.3	43.7	830		781	
Brain†.....	33.5	31.0	33.4	786	795	780	6.2	4.1	69.7	67.3	75.0	33.3	30.8	33.3	790	799	785	
Perirenal fat.....		12.8	12.1		120	95		4.0			825.0	863.0	72.6	88.6		683	690	
Heart.....	28.5	24.4	25.2	789	768	783	96.0	117.0	89.5	5.6	5.4	22.3	15.4	19.2	798	773	786	
Abdominal muscle.....	18.3	17.4	19.4	762	757	758	4.5	4.4	10.6	13.7	22.0	20.2	18.4	17.5	19.1	776	775	
Gastrocnemius muscle.....	11.8	14.7	19.3	758	765	764	5.7	3.8	9.6	18.1	7.5	11.5	14.6	19.2	765	780	769	
Muscle fascia.....	44.1	58.1		643	560		0	0	22.4			45.1	58.1		657	560		
Tendon.....		74.2		492			0	0	0	0	5.7	0		74.7		495		
Whole blood.....		93.8	88.7		782													
Serum.....		116.4	110.4			919												

* Blood of spleen assumed to contain 75 per cent red cells.

† Neutral fat content assumed to be 0.5 per cent instead of figures quoted.

where S , E , C , and T represent serum, extracellular phase, cells, and whole tissue respectively; brackets represent milliequivalents per kilo of water; and parentheses represent milliequivalents or gm. per kilo of tissue (minus its contained blood and fat).

Corresponding calculations of (E) , (C) , and $(H_2O)_c$ may be carried out, on the assumption that the sodium is entirely extracellular, by starting with the equation, $[Na]_t = 0.95 \times [Na]_s$. It should be noted that the calculations given above yield minimum values of (E) and $(H_2O)_c$. Manery *et al.* (19) have previously discussed the reasons for believing that the extracellular phase of muscle is a form of connective tissue, diluted with ultrafiltrate of serum. To the extent that the extracellular phase contains connective tissue proteins, the estimated values of (E) and $(H_2O)_c$ are too low. Since data are not available which permit us to make this correction for the connective tissue proteins in the tissues studied, our results will be presented in terms of (E) and $(H_2O)_c$ as defined above. The subsequent discussion will center about the calculated values of (E) , (C) , and $(H_2O)_c$.

Methods

Nineteen different tissues of eight adult albino rats and thirty-one tissues of two adult rabbits were analyzed. The animals were prepared by decapitation and bleeding, thus obviating alterations in permeability due to an anesthetic. The blood obtained was heparinized and, in most cases, both plasma and whole blood analyzed for sodium, chloride, potassium, and water. The tissues were analyzed for blood, fat, water, chloride, sodium, and, in some instances, potassium. Since a general account of the treatment of the tissues and the methods used has already been described (Manery *et al.* (19)), only certain additional details will be included here. The average difference between duplicate chloride analyses was 2.4 per cent (P.E. 2.2 per cent), sodium 3.1 per cent (P.E. 2.3 per cent), potassium 2 per cent (P.E. 1.4 per cent), water 1.2 per cent (P.E. 1.2 per cent).

Sodium—The method of Butler and Tuthill (5) was used for the determination of sodium. Although the quantity of tissue used for analysis usually contained approximately 0.1 milliequivalent of sodium, it was sometimes necessary to carry out the determination on smaller quantities. A series of observations on known

TABLE III
Analyses of Tissues of Two Rabbits

Rabbit No	Chloride			Sodium			Water			Blood*			Fat*			Chloride			Sodium			Water		
	Per kilo fresh tissue									Per kilo blood and fat-free tissue†														
	1	2	m. eq.	1	2	m. eq.	1	2	gm.	1	2	cc.	1	2	gm.	1	2	m. eq.	1	2	m. eq.	1	2	gm.
Skin.....	70.4	74.6	77.4	81.8	687	670	0	0	9.9	0	0	0	62.9	0	0	71.0	74.6	78.1	81.8	694	670	736	617	598
Subcutaneous tissue.....	53.2	63.0*	63.0*	690	690	690	0	0	62.9	0	0	0	0	0	0	56.8	76.8	67.2	72.9	617	736	617	598	
Tendon.....	76.8	73.5*	73.5*	72.6*	617	596	0	0	0	0	0	0	0	0	0	76.8	73.8	72.9	72.9	617	736	617	598	
Lung.....	67.3	63.6*	72.3*	71.5*	812	790	380.5	262.5	16.1	22.1	57.7	55.4	60.8	62.6	854	64.4	62.0	72.7	75.3	804	785	804	819	
Kidney.....	64.6	62.5	72.8	75.4	799	773	24.7	53.5	6.0	16.6	64.4	62.0	72.7	75.3	804	53.1	59.2	47.7	867	851	867	851	851	
Testes.....	52.9	58.7*	58.7*	47.5	857*	844	11.3	5.6	11.0	10.1	53.1	59.2	47.7	867	851	53.1	59.2	47.7	867	851	867	851	851	
Ovaries†.....	38.2	50.7	50.7	720	720	720	0	0	0	0	0	0	0	0	0	53.1	59.2	47.7	867	851	867	851	851	
Ear cartilage.....	56.3	70.3	148.3*	157.6	656	592	3.5	15.7	11.3	6.6	56.8	70.4	150.1	159.7	663	56.8	70.4	150.1	159.7	663	593	663	593	
Spleen.....	43.4	41.7*	41.7*	32.4*	794*	785	74.5	72.2	4.4	4.4	41.1	39.1	41.1	805	793	41.1	39.1	41.1	805	793	805	793	793	
Liver.....	27.4	31.1	33.3	33.9	740	713	66.3	25.8	2.6	2.0	23.2	29.5	29.8	32.1	738	23.2	29.5	29.8	32.1	738	712	738	712	
Stomach.....	56.1	32.6	32.6	802	802	802	5.9	5.9	27.5	27.5	57.5	47.7	33.1	825	810	57.5	47.7	33.1	825	810	825	810	810	
“ muscle.....	47.4	47.4	47.4	32.9	800	800	8.9	8.9	12.2	12.2	47.7	47.7	32.7	810	810	47.7	47.7	32.7	810	810	810	810	810	
Pyloric mucosa.....	66.1*	66.1*	66.1*	29.1*	849	849	2.2	2.2	4.3	4.3	66.4	66.4	29.1	853	853	66.4	66.4	29.1	853	853	853	853	853	
Fundic “.....	76.3*	76.3*	76.3*	36.7*	795	795	17.4	17.4	13.7	13.7	77.2	77.2	36.0	806	806	77.2	77.2	36.0	806	806	806	806	806	
Small intestine.....	43.4	46.5	50.0	53.0	825	787	11.0	3.7	6.5	7.4	43.1	46.8	49.8	793	793	43.1	46.8	49.8	831	793	831	793	793	
Cecum.....	24.6	24.3*	40.8	53.0	871	795	0.7	3.4	2.8	24.4	24.7	40.9	54.2	815	815	24.7	40.9	54.2	873	815	873	815	815	
Colon.....	43.9*	43.9*	43.9*	56.1	748	748	0	0	44.3	44.3	45.8	45.8	58.5	783	783	45.8	45.8	58.5	783	783	783	783	783	
Sciatic nerve.	50.2*	50.2*	50.2*	77.7*	587*	587*	0	0	215.0	215.0	50.5	50.5	78.0	590	590	50.5	50.5	78.0	78.0	590	590	590	590	
Spinal cord.....	42.3*	40.7*	62.5*	62.0*	702	668*	2.4	8.9	215.0	215.0	42.4	40.4	62.8	670	670	42.4	40.4	62.8	61.9	705	670	670	670	
Brain, gray.....	39.7*	52.1*	52.1*	812*	812*	812*	13.9	13.9	0	0	39.2	39.2	51.8	815	815	39.2	39.2	51.8	815	815	815	815	815	

	36.5*		755*	13.7	123.8	35.9			757
Brain, white.....									
Cerebrum + medulla oblongata.....		46.5	59.9*		7.9		46.4	59.9	777
Cerebellum.....		46.7	63.0*				46.7	63.0	792
Heart.....	38.0*	38.5	52.6*						808
Diaphragm.....	27.4*	28.7*	32.0*775*762*	49.0	67.5	20.8	37.9	51.5	783
Abdominal muscle.....	19.5*	18.3	26.3	28.4	33.6	26.8	26.4	30.4	779
Gastrocnemius.....	13.7	14.6	16.3	8.5	9.6	42.8	19.7	27.4	760
Mesentery.....	9.8*	12.7	19.2*	7.4	15.4	6.7	13.2	15.8	745
Perirenal fat.....		7.4		9.3	4.4	817.0	51.9	104.4	800
Blood.....	88.5	93.4	96.9*104.2*830*819		3.5		147.0		
Plasma.....	103.1	110.4*	142.7						

* The figures represent single analyses. All others are averages of duplicates.

† A neutral fat content of 0.5 per cent has been assumed for all nervous tissue instead of that quoted. The blood of the colon and cerebellum of Rabbit 2 was assumed to be 3.4 and 7.9 cc. respectively and the neutral fat of the diaphragm of Rabbit 2 to be 26.8 gm.

‡ The figures are averages of ovary analyses of three to six rabbits.

solutions demonstrated that as little as 0.04 milliequivalent of sodium can be determined within the experimental error of 3 per cent. It was found that the use of mechanical stirring for 8 minutes during the formation of the precipitate was essential to achieve this accuracy with small amounts of sodium.

The presence of potassium leads to high results for sodium when the ratio K:Na is greater than 2. A series of determinations was carried out in which the sodium present was 0.1 milliequivalent and the K:Na ratio was varied from 0 to 10. From these data, an empirical correction was applied to our sodium determinations of tissues in which the K:Na ratio exceeded 2. Specifically, this amounted to the subtraction of 3 per cent from the apparent sodium values of liver and spleen, and of 5 per cent from those of skeletal muscle.

Chlorides—Chloride determinations were carried out in 50 cc. short, conical, Pyrex centrifuge tubes. Almost complete decolorization with chloride-free potassium permanganate before titration and centrifuging just prior to the end-point (Fiske and Sokhey (8)) were adopted. The use of excessive amounts of oxalic acid to decolorize permanganate must be avoided, since it completely masks the titration end-point. An optimum concentration of nitric acid (3 cc. of concentrated HNO_3 per 10 cc.) is also essential for a stable end-point.

Blood and Fat—In order to make comparisons between tissues of such diversity as those studied here, it was necessary to express the constituents in terms of blood-free, fat-free tissue. There are, however, certain errors in the application of the blood and fat corrections which deserve special mention. The determination of blood depends on the comparison of the amount of hemoglobin in the tissue with that in the whole blood of the same animal. This implies that the concentration of red cells in the tissue capillaries is the same as that in the circulating blood. Barcroft and Poole (2) reported that the blood of the splenic pulp was 50 per cent richer in red cells than the blood from large vessels. This degree of enrichment of the splenic blood has been assumed in our experiments in correcting for the blood content of the spleen.

The method of extracting neutral fat with ether, as described by Hastings and Eichelberger (12), leads to high results for nervous

tissues. We have, therefore, applied to brain and nerve the value 0.5 per cent found by Bloor (3) for the neutral fat of brain. Our values for neutral fat in liver, lung, kidney, heart, and skeletal muscle are in essential agreement with those of Bloor *et al.* (3, 4). Since the calculations are not affected by fat corrections of less than about 5 per cent, the corrections are negligible in most tissues. However, they have been applied routinely for the sake of consistency.

Results

Chloride versus Potassium—In muscle, the chloride and most of the potassium are believed to be confined to separate phases. If the composition of the intra- and extracellular phases were constant from tissue to tissue, but varied only in their relative proportions, then, in a series of tissues of different composition, a reciprocal relation should exist between the intracellular potassium and the extracellular chloride, expressed in units per kilo of tissue water. No such reciprocal relation is apparent when one compares the uncorrected analytical values of chloride and potassium of different tissues (Table I). However, when corrections are applied for the chloride and potassium of the blood contained in the tissue, and for the potassium present in the extracellular fluid, an approximate inverse relation between these tissue constituents appears (Fig. 1). The points of Fig. 1 were calculated from data given in Table I. Fig. 1 indicates that, in a general way, tissues with high potassium contain a low chloride concentration and *vice versa*. This may be regarded as support for the thesis that chloride is confined to the extracellular phase and that potassium is concentrated within cells. However, deviations from such a simple relation, which are outside the limit of experimental error, point to the inadequacy of the hypothesis as a complete quantitative description.

Extracellular Phase—From the data given in Tables II and III, the magnitude of the extracellular phase, (*E*), of the different tissues of the rat and rabbit has been calculated by the method described above. These data are presented in Table IV. The values shown in the first three columns are based on the assumption that all of the chloride was extracellular, and in the last two, that all of the sodium was extracellular. The tissues are listed in

the order of increasing values of (E) for the rat. The values of (E) for most of the corresponding tissues of the rabbit are similar in magnitude. Such calculations reveal variations in (E) from 11 per cent for skeletal muscle to 100 per cent for certain connective tissues. Those having values for the extracellular phase of 35 per cent or less are the skeletal muscles, heart, liver, spleen, brain, spinal cord, and ovaries. The gastrointestinal tract, testes,

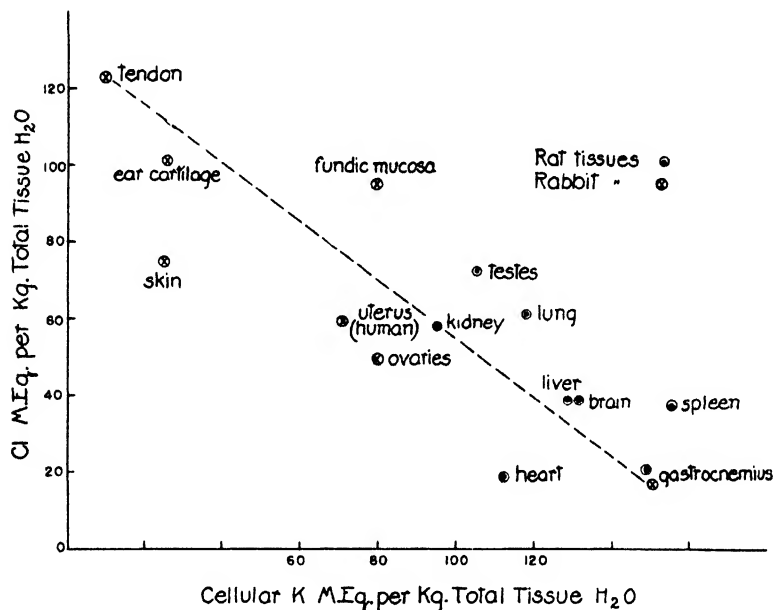


FIG. 1. The K of the intracellular phase in milliequivalents per kilo of tissue water is plotted against the tissue Cl, in milliequivalents per kilo of tissue water. The values have been calculated from the data of Table I, corrections having been made for the Cl and K of the blood contained in the respective tissues and for the K of the extracellular phase.

lung, and kidney yield values of (E) ranging as high as 62 per cent for the gastric mucosa.

Basing the calculation of the extracellular phase on the sodium instead of the chloride does not materially alter the magnitude of (E) in most tissues; it is significantly larger in the ear cartilage, and definitely smaller in the lung and testes, all parts of the stomach, and in most connective tissues other than cartilage. It is

obvious from the data of Table IV that the assumption that sodium and chloride are both entirely extracellular in all tissues is not justifiable. The physiological interpretation of these discrepancies will be discussed in a later section.

TABLE IV

Apparent Extracellular Phase (E) Calculated from Cl and Na (Gm. per Kilo of Blood-Free, Fat-Free Tissue)

	Chloride			Sodium	
	Rats (3 to 8)	Rabbit 1	Rabbit 2	Rabbit 1	Rabbit 2
Gastrocnemius	119	117	109	112	108
Abdominal muscle.....	143	173	153	193	181
Diaphragm		233	220		198
Heart.....	140	320	306	364	350
Liver	219	205	238	210	209
Spleen	276	364	316		268
Brain	255	332	376	366	400
Spinal cord		375	324	443	403
Ovaries*		322		341	
Large intestine	316		369		381
Small "	400	381	378	352	354
Testes	499	470	478		311
Lung	423	510	447	430	408
Kidney	399	569	502	513	490
Stomach†	509	508	386	233	213
Pyloric mucosa.....			535		190
Fundic "			624		234
Mesentery		458	540	737	
Subcutaneous tissue....	579	502		475	
Ear cartilage		502	567	1060	1040
Skin		627	603	552	532
Tendon	568	683‡	595	552‡	473
Perirenal fat	623		1185		

* Average of three to six rabbits. No corrections.

† The figures for rats and Rabbit 1 apply to whole stomach; those for Rabbit 2 to stomach muscle.

‡ Average of thirteen rabbits.

Intracellular Water—The values of $(H_2O)_c$, the intracellular water, calculated according to the equations given in an earlier section are presented in Table V. It is believed that intracellular

water values ranging from 70 to 75 per cent may be regarded as reasonable values. The skeletal muscles, which include the diaphragm, and the abdominal and gastrocnemius muscles of the rat and rabbit, have values of $(\text{H}_2\text{O})_c$ which vary only from 725 to 754 gm. per kilo of cells. The value of $(\text{H}_2\text{O})_c$ for the heart is also essentially the same as that found for the skeletal muscles. When one considers the values of $(\text{H}_2\text{O})_c$ for other tissues, one encounters some palpably unreasonable figures. This is particularly true of the connective tissues where negative values for $(\text{H}_2\text{O})_c$ were sometimes found.

Between these two extreme conditions, the muscles, where $(\text{H}_2\text{O})_c$ is entirely reasonable, and the connective tissues, where calculated values of $(\text{H}_2\text{O})_c$ are quite unreasonable, lie a large number of tissues whose calculated intracellular water varies between 50 and 70 per cent.

The criteria used for this calculation seem to be inapplicable to connective tissues and therefore invalid for each organ to a degree which depends on the amount of connective tissue it possesses. We have previously reported the observation that muscle fascia contains a high concentration of chloride and have concluded that the extracellular phase of muscle should be regarded as closely related to the connective tissue phase (18, 19). We are now led to the same conclusion with regard to all the tissues which we have studied—namely, that the extracellular phase resembles connective tissue more closely than a serum ultrafiltrate and should be considered to include connective tissue proteins. Applying this consideration to muscle led to the conclusion that its extracellular phase contains 22 per cent solids rather than 1 per cent (19). A similar correction made for the tissues under discussion would have the effect of increasing their calculated intracellular water concentration.

Sodium versus Chloride—The extracellular position of sodium and chloride in skeletal muscle has received support from the observation that the ratio Na:Cl is essentially the same in muscle as in a serum ultrafiltrate. By plotting the values for sodium and chloride of rabbit tissues, one may show the extent to which the Na:Cl ratios conform to that of an ultrafiltrate (Figs. 2 and 3). A line has arbitrarily been drawn from the point representing the ultrafiltrate to the origin. Many points cluster about this line,

showing that an essentially linear relationship exists between their sodium and chloride concentration and that the ratio of Na:Cl approximates that of an ultrafiltrate. The tissues showing this consistency are abdominal muscle, diaphragm, liver, spleen, intestine, brain, and kidney. They differ in apparently having a greater proportion of extracellular phase (Table V), the greater the distance of the points from the origin. The points close to

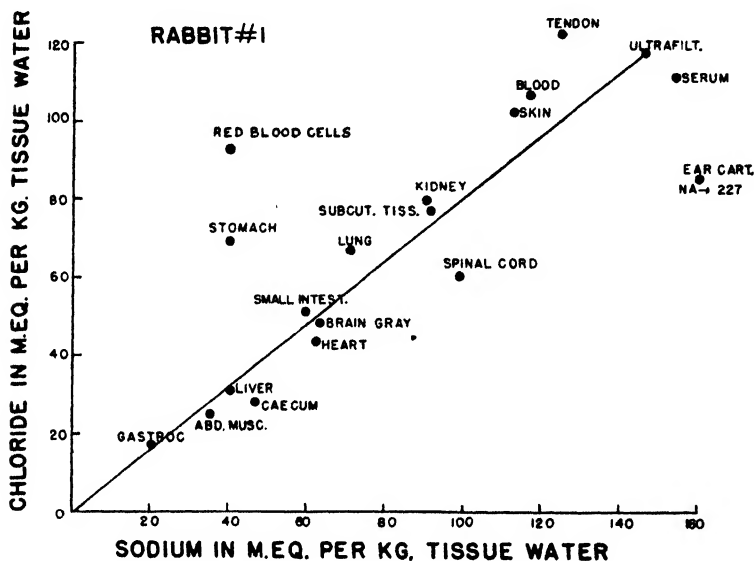


FIG. 2. The tissue sodium, in milliequivalents per kilo of tissue water, is plotted against the tissue Cl, in milliequivalents per kilo of tissue water, for Rabbit 1. The values have been calculated from the data of Table III. Corrections have been made for the Na and Cl of the blood contained in the respective tissues.

the line represent tissues which possess a phase containing sodium and chloride in approximately serum proportions; those removed from the line on the left represent tissues containing cells in which the intracellular chloride exceeds the intracellular sodium, and those on the right, in which intracellular sodium exceeds the chloride.

From these observations, the following tentative conclusions may be drawn:

1. The tissues whose points lie close to the line consist of (a) cells, similar to muscle cells, which are free of and impermeable to sodium and chloride, and (b) another phase which contains both sodium and chloride in serum ultrafiltrate proportions. This latter phase may be similar to connective tissue, and conceivably may include cells which contain both sodium and chloride, but are permeable only to one. For reasons pointed out by Wu (21),

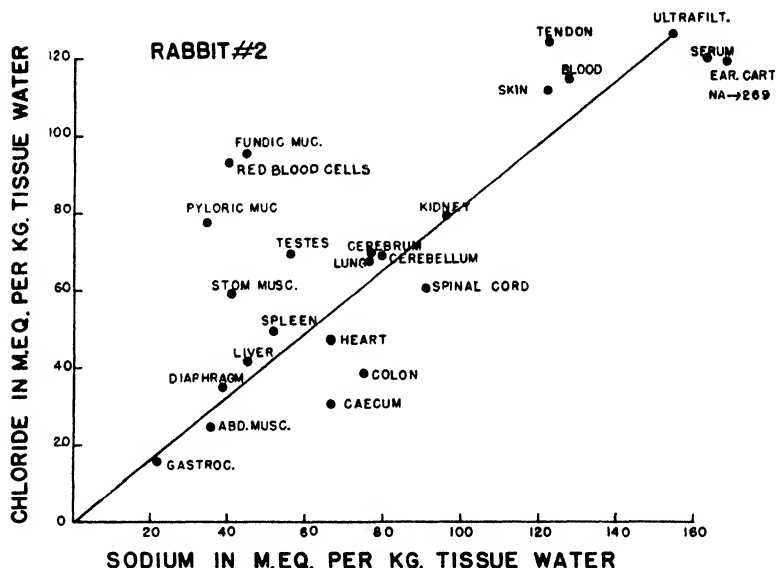


FIG. 3. The tissue sodium, in milliequivalents per kilo of tissue water, is plotted against the tissue Cl, in milliequivalents per kilo of tissue water, for Rabbit 2. The values have been calculated from the data of Table III. Corrections have been made for the Na and Cl of the blood contained in the respective tissues.

it is unlikely that mammalian cells will be found to be impermeable to proteins and permeable to both cations and anions.

2. The tissues whose points lie to the left of the line contain, in addition to the phases already mentioned, some cells which are relatively high in chloride and low in sodium. Such cells might resemble red blood cells in their composition and permeability properties.

3. The tissues whose points lie to the right of the line contain,

in addition to the phases mentioned in conclusion (1), some cells which are relatively high in sodium and low in chloride.

The tissues whose values deviate from the line will now be discussed in greater detail. The blood requires no special explana-

TABLE V
Intracellular Water (Gm. per Kilo of Cells)

	Rats 1 to 5	Rat 6	Rat 7	Rat 8	Rabbit 1	Rabbit 2
Testes.....	765	768	775	750	748	723
Heart.....	766	757	744	748	738	727
Abdominal muscle.....		740	741	735	720	740
Gastrocnemius..	738	742	754	729	747	731
Diaphragm....					734	725
Spleen	738	741	734	726	693	702
Brain	720	720	740	703	681	661
Lung	720	669	735		702	680
Liver.....	653	625	651	625	670	633
Kidney.....	699	627	612		544	600
Large intestine..		767		669		660
Small " ..			573	521	733	672
Stomach*.....		584	560	445	644	698
Pyloric mucosa.						695
Fundic " ..						503
Ovaries†.....					575	
Spinal cord					536	500
Mesentery.....					537	537
Subcutaneous tissue.....		522	601	539	470	
Ear cartilage....					323	69
Perirenal fat ...			294	Negative		Negative
Muscle fascia		477	220			
Skin.....		288	349	282	180	184
Tendon.....			Negative		Negative‡	20

* The figure for Rabbit 1 applies to whole stomach; that for Rabbit 2 to stomach muscle.

† Average of three to six rabbits. No corrections.

‡ Average of thirteen rabbits.

tion, since the erythrocytes contain and are freely permeable to chloride, but are impermeable under normal conditions to sodium, and in many species, contain a relatively small amount of it.

Stomach was found to contain much more chloride than sodium. This is hardly surprising in view of its function of secreting hydrochloric acid. In Rabbit 1 (Fig. 2), the analysis of the entire stomach wall exhibits considerable excess chloride over sodium. In Rabbit 2 (Fig. 3), the mucosa was separated from the muscle, presumably at the submucous layer, and both the fundic and pyloric mucosae were analyzed. The fundic mucosa, where the parietal cells are concentrated, contains the highest chloride content and, therefore, a high extracellular space (Table V). This is in conformity with the observations of Linderstrøm-Lang and Holter (15) that the fundic mucosa is the site of greatest localization of acid. The high chloride concentration in the mucosa is no doubt largely due to the presence of chloride in the foveolae of the glands and on the surface epithelium, since it has been observed there in large amounts (Lison (16) and Gersh (10)). The histochemical methods of these investigators demonstrated its presence only in connective tissue and in the zymogen cells, but not in other gland cells. The values for the extracellular phase of stomach and its parts calculated from our chloride analyses are unreasonably high. Those calculated from the sodium analyses are entirely reasonable, however. This does not prove the permeability of cells to chloride, but the formation of the hydrochloric acid of the gastric juice would be difficult to explain if it were otherwise.

It has previously been shown (19) that dense and probably loose connective tissues have chemically equivalent amounts of sodium and chloride. Hence, tendon and skin would not be expected to have the same Na:Cl ratio as that of an ultrafiltrate. The reason for the excess chloride in lung and testes is a matter of conjecture, but both are known to contain large amounts of connective tissue. In the testis, the capsule, mediastinum, and septula are composed of dense connective tissue, and the interstitial cells situated between the lobules are regarded as modified connective tissue cells. Large amounts of connective tissue proteins can be observed in the walls of the respiratory bronchioles of the lung, and some investigators consider the alveolar walls to be of connective tissue origin.

Cartilage contains a large excess of sodium, a fact which was inferred by Logan (17) from the analysis of the bases of cartilage.

Iob and Swanson (14) determined sodium directly, and found, as we have, that the sodium greatly exceeds the chloride content. This sodium is not in solution in the aqueous phase, but is probably a part of the organic matrix.

The fact that chloride and sodium are known to be employed in the stomach and cartilage for purposes other than those associated with the maintenance of a constant extracellular environment lends added support to the hypothesis that most of the sodium and chloride of tissues is in an extracellular phase which is in ionic equilibrium with the blood plasma.

DISCUSSION

It is pertinent to inquire to what extent the data presented contribute information which aids in answering the questions raised at the beginning of this paper.

The evidence presented indicates that, for the purposes of an approximate description of tissues, at least three chemically different phases are necessary. These phases which likewise have morphological significance are: (1) an extracellular phase, $(E)_p$, which is in ionic (Donnan) equilibrium with blood plasma and consists essentially of plasma ultrafiltrate and connective tissue proteins; (2) an intracellular phase, $(C)_1$, which contains neither sodium nor chloride and is exemplified by muscle fibers; and (3) an intracellular phase, $(C)_2$, which contains chloride and may or may not have sodium in equivalent proportions; *e.g.*, blood cells and connective tissue cells. The relative proportions of these three phases in different tissues of the rabbit cannot, at present, be expressed in quantitative terms except in a few instances.

The connective tissues studied consist almost entirely of the extracellular phase, $(E)_p$, but have, in addition, a small intracellular phase $(C)_2$. The presence of the connective tissue proteins, such as exist in tendon, exerts slight, if any, influence on the ionic pattern of the tissue (19).

The skeletal muscles and the heart can be adequately described in terms of the two phases, $(E)_p$ and $(C)_1$, although they, too, probably contain a small amount of the phase, $(C)_2$. The extracellular phase of the skeletal muscle varies from 10 to 20 per cent depending upon the amount of connective tissue present. The

extracellular phase of the rabbit heart is between 30 and 35 per cent. In the rat, it is much lower.

Other tissues in which the sodium and chloride are present in ultrafiltrate proportions are the liver, spleen, intestine, brain, lung, and kidney. In view of the complex nature of these tissues, it would be unreasonable to interpret this as evidence that they are simple mixtures of the phases, $(E)_p$ and $(C)_1$. It would rather be more consistent with the histological composition of these tissues to regard the calculated extracellular phase (E) , which is the total chloride space, as actually representing the sum of the extracellular phase $(E)_p$ plus an intracellular phase, $(C)_2$, containing Na and Cl in approximately the same proportions as $(E)_p$. It is not possible at this time to estimate the relative proportions of $(E)_p$ and $(C)_2$. All that one might say is that $(C)_2$ is probably low in liver and high in kidney.

Examples of tissues which do not contain sodium and chloride in ultrafiltrate proportions are the stomach and testes. In these tissues, the values of the extracellular phase (E) calculated from sodium are reasonable, those calculated from chloride quite unreasonable. It seems necessary to assume that these tissues contain, in addition to $(E)_p$ and $(C)_2$, an intracellular phase containing an excess of chloride over sodium and may or may not contain an intracellular phase $(C)_1$.

CONCLUSIONS

The data presented show that living mammalian tissues cannot be divided into intra- and extracellular phases in the same manner as muscle. There are at least two groups: (a) tissues which have a large proportion of chloride-free cells, skeletal muscles, liver, spleen, heart, brain, and kidney; (b) tissues which have a large proportion of chloride-containing cells, blood, connective tissues, gastric mucosa, testes, and probably lung.

These conclusions are based on the following considerations.

When the extracellular phase of tissues was estimated on the assumption that all of the chloride was extracellular, the magnitude of the phase varied from 11 per cent to 35 per cent in the tissues of group (a), but it far exceeded this magnitude in the tissues of group (b).

The intracellular water, calculated on the assumption that the

extracellular phase is a serum ultrafiltrate, was very low in some of the tissues in group (b). This emphasized the fact that connective tissue proteins should be included in estimating the magnitude of the extracellular phase.

The Na:Cl ratios of the tissues of group (a) are the same as in an ultrafiltrate, indicating the extracellular position of sodium and chloride in a large portion of the tissue. The tissues in group (b) contain chloride in excess of sodium which is interpreted as indicative of intracellular chloride.

SUMMARY

1. A direct relation is found to exist between the chloride and sodium, and an inverse relation between the chloride and potassium of many mammalian tissues, when allowance is made for their content of blood and fat.

2. These observations have been interpreted in terms of the relative masses of extra- and intracellular phases of the tissues.

3. Exceptions to the extracellular position of sodium and chloride have been noted and the significance of these exceptions discussed.

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THE DIETARY INDISPENSABILITY OF VALINE*

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Biochemical literature records no reliable information concerning the relation of valine to growth. Since the discovery of this amino acid in biological materials (von Garup-Besanez, 1856), little has been added to our knowledge of its metabolic significance. Doubtless this is to be accounted for, as in the case of several other amino acids, by the fact that no procedure exists whereby valine may be removed quantitatively from the products of protein hydrolysis. In order to determine its nutritive rôle, one must resort to the use of diets in which the nitrogen is supplied in the form of mixtures of highly purified amino acids. Such experiments have now been consummated. The results demonstrate that valine, despite its relative simplicity in structure, cannot be synthesized by the organism of the rat. Animals deprived of it experience a profound nutritive failure, with rapid decline in weight, loss of appetite, and eventual death. Furthermore, they manifest strange symptoms which appear to be characteristic of this type of dietary inadequacy. The details of the methods employed and of the results obtained are outlined below.

EXPERIMENTAL

The composition of the amino acid mixture (Mixture XV-b) is shown in Table I. As will be observed, alanine, valine, and

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The results of this investigation were presented in abstract (Rose, 1937, 1938).

† The experimental data in this paper are taken from a thesis submitted by Samuel H. Eppstein in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate School of the University of Illinois.

threonine were not included. Alanine was omitted inasmuch as the mixture was designed for use in another investigation involving this amino acid. The three missing components were introduced directly into the diets in the quantities desired. All

TABLE I
Composition of Amino Acid Mixture

	Mixture XV-b	
	Active amino acids	As used
	gm.	gm.
Glycine.....	3.00	3.00
Alanine.....	0	0
Valine.....	0	0
Leucine.....	9.00	18.00*
Isoleucine.....	4.00	8.00*
Norleucine.....	1.25	2.50*
Proline.....	8.00	8.00
Hydroxyproline.....	2.00	2.00
Phenylalanine.....	3.90	7.80*
Glutamic acid.....	22.00	22.00
Aspartic ".....	4.10	4.10
Serine.....	1.50	3.00*
Tyrosine.....	6.50	6.50
Cystine.....	1.25	1.25
Histidine.....	3.70	
" monohydrochloride monohydrate.....		5.00
Arginine.....	5.25	
" monohydrochloride.....		6.35
Lysine.....	7.70	
" dihydrochloride.....		11.55
Tryptophane.....	2.25	2.25
Methionine.....	1.75	3.50*
Sodium bicarbonate.....		13.38
	87.15	128.18†

* Racemic acids.

† 1.471 gm. of the mixture are equivalent to 1.0 gm. of active amino acids.

of the amino acids were shown to be analytically pure before being incorporated in the food.

The make-up of the diets is shown in Table II. Each supplied 18 per cent of active amino acids including glucosamine, and was

administered *ad libitum*. Diet 1 contained 4.0 per cent of *dl*-valine. Undoubtedly, this amount is far in excess of the needs of the organism, but at the time the experiments were conducted no information was available as to the optimum valine intake. Diet 2 differed from Diet 1 in that it was devoid of valine. The vitamin B factors were furnished to each animal in the form of two pills daily, each containing 75 mg. of milk concentrate and 50 mg.

TABLE II
*Composition of Diets**

	Diet 1	Diet 2
	gm.	gm.
Amino acid Mixture XV-b.....	20.6	23.5
Alanine (<i>dl</i> -).....	1.0	1.0
Threonine (<i>d</i> -)†.....	0.7	0.7
Glucosamine hydrochloride (<i>d</i> -).....	1.0	1.0
Sodium bicarbonate.....	0.4	0.4
Dextrin.....	20.3	21.4
Sucrose.....	15.0	15.0
Salt mixture‡.....	4.0	4.0
Agar.....	2.0	2.0
Lard.....	26.0	26.0
Cod liver oil.....	5.0	5.0
Valine (<i>dl</i> -).....	4.0	0
	100.0	100.0

* Each diet contained 18 per cent of active amino acids, including glucosamine. The vitamin B factors were supplied in the form of two pills daily, each containing 75 mg. of milk concentrate and 50 mg. of tikitiki extract. The daily intake of nitrogen from these sources amounted to approximately 4 mg.

† Natural *d*(-)-threonine (*cf.* Meyer and Rose (1936)).

‡ Osborne and Mendel (1919).

of tikitiki extract. The daily intake of nitrogen from these sources amounted to slightly less than 4 mg., and was the only nitrogen of unknown kind in the rations.

The results of the experiments are summarized in Charts I and II. In Table III are recorded the total changes in body weight and the total food intakes of the animals. All of the members of Litter 1 (Chart I), with the exception of Rat 2457,

received the valine-free diet for a period of 28 days. During this time they manifested a profound nutritive failure, with rapid

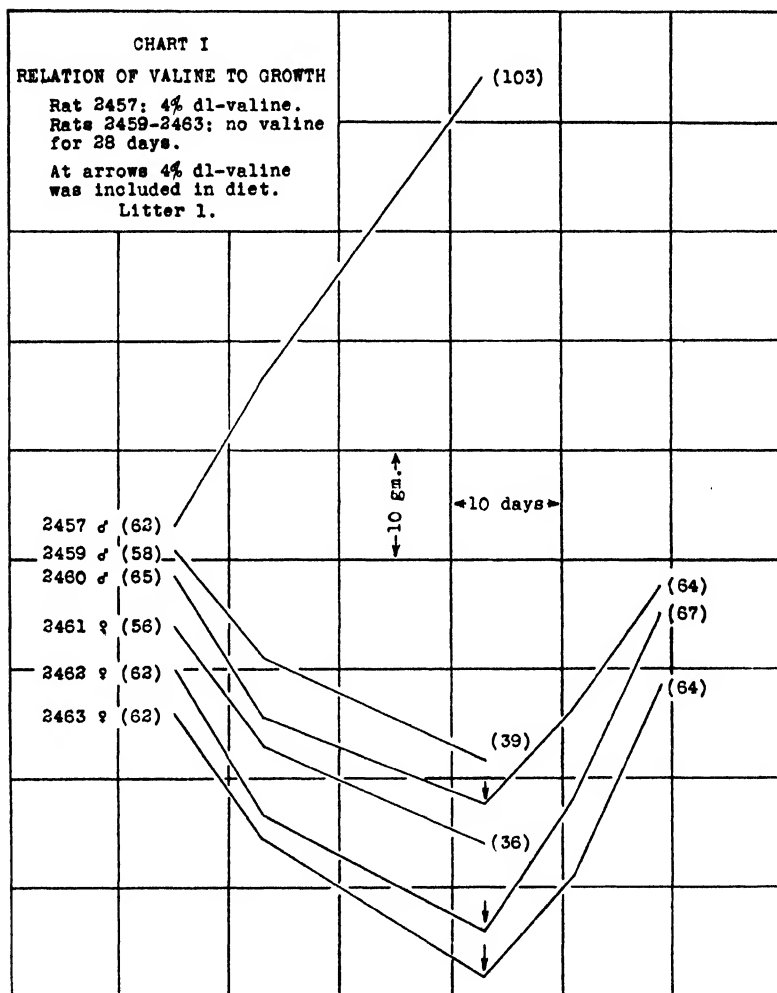


CHART I. The numbers in parentheses denote the initial and final weights of the rats.

decrease in weight and marked loss of appetite. Three animals were then transferred to the ration containing valine. Growth

promptly ensued. Of Litter 2 (Chart II), three members received valine and two were deprived of it for the duration of the test.

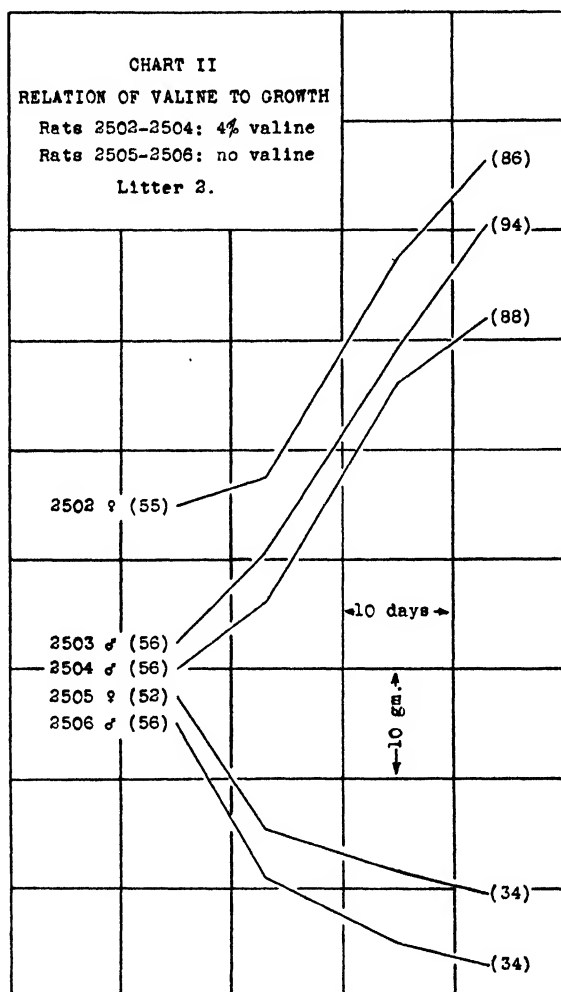


CHART II. The numbers in parentheses denote the initial and final weights of the rats.

The difference in growth behavior of the animals which received the amino acid, as contrasted with those which did not, is quite

striking. Similar experiments were conducted upon four additional litters, but inasmuch as the results were quite uniform, the details are omitted from this report.

The most striking feature of valine deprivation is the development of peculiar symptoms which are unlike any we have encountered in other types of amino acid deficiencies. The rats become extremely sensitive to touch, and display a severe lack of coordination in movement. They walk with a staggering gait. The initial stages of this condition are illustrated in Fig. 1. As the

TABLE III
*Total Changes in Body Weight and Total Food Intakes of
Experimental Animals*

Litter No.	Rat No. and sex	Days	Total change in weight	Total food intake	Supplement
			<i>gm.</i>	<i>gm.</i>	
1	2457 ♂	28	+41	153	4% <i>dl</i> -valine
	2459 ♂	28	-19	56	No valine
	2460 ♂	28	-21	57	" "
		16	+20	55	4% <i>dl</i> -valine
	2461 ♀	28	-20	56	No valine
	2462 ♀	28	-24	53	" "
		16	+29	58	4% <i>dl</i> -valine
	2463 ♀	28	-24	58	No valine
		16	+26	55	4% <i>dl</i> -valine
					" "
2	2502 ♀	28	+32	115	" "
	2503 ♂	28	+38	125	" "
	2504 ♂	28	+32	123	" "
	2505 ♀	28	-18	49	No valine
	2506 ♂	28	-22	38	" "

animal attempts to walk, the left fore leg is raised inordinately, and the head is retracted. Frequently, the subjects show a rotary motion resembling that of a dog chasing his tail. This may be either clockwise or counter-clockwise, and may continue until the animals fall to the floor of the cage from sheer exhaustion. As would be anticipated, the symptoms are readily cured by the administration of valine without any other therapeutic measure. The remarkable effects of the amino acid are dramatically portrayed in Fig. 2 (Rat 2460). The upper photograph was taken on the 28th day of the deficiency. At that time, the subject had

lost one-third of its body weight, was too weak to consume food, and appeared to be at the point of death. Valine was administered several times in solution by pipette. The next day the



FIG. 1. Rat 2461 ♀, showing the initial stages of the incoordination in movement observed in animals deprived of valine.



FIG. 2. The upper photograph shows Rat 2460 on the 28th day of valine deprivation. The lower photograph shows the same animal after valine had been administered for 25 days.

animal showed marked improvement and was able to eat. The amino acid was then incorporated in the basal ration. The lower photograph was taken 24 days later. All symptoms had dis-

appeared, the fur coat had become smooth, and the body weight had increased from 44 gm. to 82 gm.

Whether the unique syndrome following valine deprivation is associated with specific lesions of the nervous system has not yet been determined. It is our intention to investigate this aspect of the problem in the near future. In the meantime, there can be no doubt that *valine is an indispensable dietary component*.

SUMMARY

By the use of diets devoid of proteins, but containing mixtures of highly purified amino acids, valine has been shown to be an indispensable component of the food.

Rats deprived of valine manifest peculiar symptoms which appear to be characteristic of this type of dietary deficiency.

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THE AMINO ACID COMPOSITION OF KERATINS

THE COMPOSITION OF GORGONIN, SPONGIN, TURTLE SCUTES, AND OTHER KERATINS

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Keratins are defined as insoluble proteins which are extraordinarily resistant to digestion by the usual proteolytic enzymes. The resistance of keratins towards enzymatic digestion seems to depend, in part, on their fibrous structure which results from the joining together of the peptide chains in a definite manner in space. If the organization of a keratin is destroyed by mechanical (1, 2) or chemical (3, 4) means, it loses this resistance to enzymatic hydrolysis. Histologically and chemically keratins are not always homogeneous proteins. Animal hairs such as wool are composed of medullary, cortical, and intercellular keratins which differ in resistance towards enzymes and in sulfur content. Reduction of wool keratin results in a soluble protein which can be fractionated by ammonium sulfate into two components (3), while grinding wool in a ball mill for prolonged periods of time yields a water-soluble fraction containing nitrogen and sulfur (2). Kühne (1) in 1877, showed that very finely divided hair is digested by pepsin. Routh and Lewis (2) have recently confirmed this observation and find furthermore that when powdered wool is extracted with water, a soluble nitrogen- and sulfur-containing compound is obtained. Recent investigations on the amino acid composition of the keratins have indicated that they may be divided into two classes, eukeratins and pseudokeratins (5). It was suggested that those keratins which are chemically similar to cattle horn (κέρας) be named eukeratins. Eukeratins are defined as insoluble proteins, resistant to enzymatic digestion, which yield histidine, lysine, and arginine in the molecular ratios of approximately 1:4:12. Those

keratins which do not yield histidine, lysine, and arginine in molecular ratios of approximately 1:4:12 have been designated, for convenience, as *pseudokeratins*. The pseudokeratins in general do not show the same resistance towards enzymatic digestion as do the eukeratins, for from 25 to 60 per cent of the pseudokeratins is often dissolved by treatment with pepsin and trypsin.¹

The experiments reported in this paper are a continuation of our studies on comparative biochemistry. The keratins from two species of *Gorgonia*, *Gorgonia flabellum* and *Plaxaurella dichotoma*, and one species of sponge, *Euspongia*, have been reinvestigated, while the external covering of the turtle shell, the scutes, and a horny excrescence which appears on the bill of the male American white pelican (*Pelicanus erythrorhynchus*) during the mating season have been studied for their amino acid composition.

EXPERIMENTAL

The procedures given below are those used in the preparation, purification, and analysis of the keratins, although minor variations in the methods were often required by the nature of the material.

The tissue was ground fine enough to pass a 20 mesh sieve. The fat was removed with hot organic solvents, and the tissue was extracted overnight at 37–40° with a large volume of 1:3 HCl. After removal of the acid by thorough washing with cold followed by warm water, the residue was digested with pepsin and HCl for 24 hours. At the conclusion of the peptic digestion, the washed residue was digested with trypsin in phosphate buffer at pH 7.8. After removal of the buffer, the residue was extracted with hot organic solvents and then dried at 110° to constant weight.

All chemical analyses were carried out two or more times, preferably on different samples of protein.

Total nitrogen was estimated by the usual Kjeldahl method with Cu and Se as catalysts. Sulfur was estimated both by the Parr bomb and the Pregl micromethod. Iodine was determined by alkali fusion, reduction of the resulting NaI to I₂, and colorimetric comparison of the latter in CCl₄ solution (6).

The basic amino acids were isolated by the silver precipitation

¹ The amino acid composition of the keratins, especially the pseudokeratins, may change somewhat during the course of purification.

method (7) and the purity of the resulting flavianates, nitranylates, and picrates was checked by analyses and melting points. Cystine was determined by two methods: colorimetrically by Miller and du Vigneaud's procedure (8) and gravimetrically by precipitation with Cu_2O according to Graff *et al.* (9). Tyrosine was estimated by a modification of Folin's method (*cf.* (7)). Tryptophane was determined by Folin's method and by both Bates' (10) and Sullivan's (11) adaptations of the dimethylaminobenzaldehyde procedure. Glycine was determined by a modification of Bergmann and Fox's method (*cf.* (7)), phenylalanine by the dinitrobenzene procedure (7), and diiodotyrosine by Lugg's method (12).

Results

Gorgonin—Valenciennes (13) first pointed out in 1855 that the internal skeleton of certain Mediterranean *Gorgonia* was composed of a protein similar to horn and called by him gorgonin. Subsequent investigations by Krukenberg (14), Mendel (15), Mörner (16), and others (17–20) suggested that these insoluble and indigestible proteins are keratins similar to those obtained from the ectoderm of the higher animals. Mörner (16) and Henze (18) reported the presence of tyrosine, arginine, lysine, leucine, histidine, phenylalanine, diiodotyrosine, glycine, alanine, aspartic acid, and glutamic acid. In one case *Primnoa lepodifera*, the former investigator isolated dibromotyrosine which had apparently replaced iodo-gorgoic acid. In spite of the relatively large amount of sulfur found in gorgonin, earlier investigators were unable to isolate cystine.

The data found on analysis of the keratin of two species of *Gorgonia* obtained from the Tortugas Islands, Florida, confirm in general the previous results of Valenciennes (13), Krukenberg (14), Mendel (15), and others (16–20) which are summarized in Table I, Columns 2, 4, and 5. The results of the present experiments on *Gorgonia flabellum* and *Plexaurella dichotoma* are given in Columns 1 and 3. Inspection of Table I shows that gorgonin from *Gorgonia flabellum* and *Plexaurella dichotoma* are quite alike in chemical composition, with the exception of iodine and possibly histidine. The samples of gorgonin from *Gorgonia flabellum* contained approximately 0.13 per cent of I_2 , while that from *Plexaurella dichotoma* yielded 0.58 per cent. The unusually large amount of

TABLE I
Amino Acid Composition of Some Keratins

The values are expressed in per cent.

Constituent	Gorgonin					Spongin	Turtle scutes	Pelican excre- cence	Whale baleen	Human skin	Neuro- keratin	Silk fibroin*	
	<i>Gorgonia flabellum</i>		<i>Plexaurella dicholoma</i>		<i>Gor- gonia casotini</i>								
	(1)	(2)	(3)	(4)	(5)								
	(6)	(7)											
Nitrogen.....	14.1	15.6	13.7	15.4		13.0	14.8	14.1	14.0	14.1	14.2	13.3	19.0
Sulfur.....	1.24	1.1	1.51	1.7		0.7	0.7	2.3	1.6	3.4	1.7	2.0	0.0
Iodine.....	0.13	1.15	0.58	0.12	7.79	0.84	1.46						
Histidine.....	0.9	0.5	0.1	0.4	+	0.2	0.0	1.8	0.9	1.0	0.8	1.1	0.07
Lysine.....	3.3	2.8	2.8	3.0	3.0	3.0	3.6	1.8	3.4	3.7	4.3	3.0	0.25
Arginine.....	4.5	6.4	4.9	5.4	4.0	4.3	5.9	4.2	5.7	6.2	6.5	4.1	0.95
Cystine.....	9.0	5.5	7.6	3.2	0	2.8	+	8.6	4.0	9.5	3.8	2.8	0.0
Tyrosine.....	13.0		13.5		5	0.8	0.0	13.1	5.7	5.0	3.4	3.8	13.2
Tryptophane.....	0		0			0.0	0.0	2.3	0.9	1.0	1.8	1.1	
Phenylalanine.....	5.7		6.5		+	3.3		5.2	4.3	2.8		4.3	
Glycine.....	15.5		13.7		6	14.4	13.9						43.8
Diiodotyrosine.....	+		+			+	4.7						
Molecular ratio of lysine to arginine.....		4:6		4:6	5:6		4:6	3:6	4:6	4:6	5:6	5:6	2:6

Some results of earlier investigators are summarized in Columns 2 (15, 20), 4 (15, 20), 5 (16-18), 7 (21-24), 10 (5), 11(25), 12 (5), and 13 (26, 27).

* Silk fibroin is not considered to be a pseudokeratin.

tyrosine in gorgonin is in marked contrast to spongin which yields approximately 1 per cent of this amino acid. Turtle scutes, silk fibroin, and pepsin also contain in the neighborhood of 13 per cent of tyrosine. Estimations of the amount of tryptophane present in gorgonin by Folin's phenol reagent indicated 5.4 per cent for gorgonin from *Gorgonia flabellum* and 4.6 per cent in gorgonin from *Plexaurella dichotoma*. However, repeated tests for this amino acid with dimethylaminobenzaldehyde indicated little or no tryptophane. Therefore, in view of the unreliability of the phenol reagent in the estimation of tryptophane in complex materials, it is tentatively suggested that gorgonin is deficient in this substance.

Block and Vickery (20) in 1931 analyzed undigested *Gorgonia flabellum* and *Plexaurella dichotoma* and found that *Gorgonia flabellum* yielded histidine, lysine, and arginine in the molecular ratios of 1:6:12 and *Plexaurella dichotoma* contained these amino acids in the molecular ratios of 1:8:12. In view of the general similarity of these ratios to that assigned to the eukeratins (1:4:12), it was tentatively assumed at that time that gorgonin may be a eukeratin. Analyses of somewhat more highly purified gorgonin preparations by the improved method for the basic amino acids yielded comparatively more histidine and lysine but less arginine than the earlier analyses and indicate that histidine, lysine, and arginine are in the molecular ratios of approximately 1:4:6 and not 1:4:12.

It was recognized in 1931 that the amounts of lysine obtained from gorgonin were somewhat high and the quantities of arginine somewhat low for a eukeratin but the histidine to arginine ratio of 1:12 was considered sufficient evidence for calling this protein a eukeratin. Since that time, other keratins have been analyzed which were characterized by a rather constant lysine to arginine ratio of approximately 4:6. These have been called pseudokeratins. The amount of histidine present in the pseudokeratins is variable. As a result of the present analyses on digested gorgonin, this protein is tentatively classed as a pseudokeratin.¹

Spongin—Sponges belong to the group of aquatic animals (*Porifera*) which are characterized by a porous structure and have either a siliceous, calcareous, or horny skeleton. The endoskeleton is derived from the dermal layer. The common bath sponge is

gathered by dredging and the digestible portions are removed by allowing the sponges to decay. The resulting fibrous skeleton, called spongin, was analyzed for C, H, N, S, I, and P, by Crooke-witt in 1843 (21). The amino acid composition of spongin has been investigated by Kossel and Kutscher (22), Abderhalden and Strauss (23), and Clancy (24). These results are summarized in Table I, Column 7. The samples used for the present analyses were obtained from a commercial source. The analytical results are summarized in Table I, Column 6. It will be seen that amounts of the basic amino acids and glycine are quite similar in gorgonin and in spongin but these pseudokeratins differ rather markedly in their content of cystine and tyrosine. The amount of cystine in spongin is low for a keratin, approximating that found in neurokeratin.

Spongin gave a color equivalent to 6.3 per cent of tryptophane with the phenol reagent but when analyzed for tryptophane by the dimethylaminobenzaldehyde and the Adamkiewicz methods, no trace of color was produced. The small amount of tyrosine present in spongin was early noted (28) and is in marked contrast to that in gorgonin, which yields about 13 per cent of this amino acid. The statement by Städeler (29) in 1859 that spongin is chemically allied to silk is extensively cited. We have, for the purposes of comparison, included in Table I some recent amino acid analyses of silk fibroin. It will be seen that the amino acid composition of spongin and fibroin is quite different. Chemically, spongin appears to be related to neurokeratin.

Scutes (Turtle)—The chemical composition of the protein of turtle scutes given in Table I, Column 8, is compiled from data obtained on duplicate analyses of three different preparations of scutes, the results of which checked closely. The amino acid values, taken together with anatomical and physiological information, seem to place tortoise scutes protein among the pseudokeratins. The chief difference between this protein and others so classified is in the larger amount of histidine (1.8 per cent) and smaller amount of lysine isolated. In contrast to other pseudokeratins (whale baleen, gorgonin, spongin, etc.) which yield over 3 per cent of lysine, less than 2 per cent was obtained from scutes although six experiments with 2.5 to 10.0 gm. of protein each were carried out. An unusual difficulty was encountered during the

lysine analyses. A considerable amount of a reddish brown, sticky precipitate appeared during the precipitation with phosphotungstic acid shortly after the appearance of the white, crystalline lysine phosphotungstate. In one experiment part of this material was removed from the centrifuge bottle without changing the yield of pure lysine picrate.

From these amino acid analyses, it appears that turtle scutes are comparatively rich in histidine and yield lysine and arginine in the molecular ratio of 3:6. This latter value is midway between the usual lysine to arginine ratios found in eukeratins and pseudokeratins. These results suggest that turtle scute protein is not a typical pseudokeratin and may be a mixture of eu- and pseudokeratins or a member of a new class of keratins.

Pelican Excrescence—The sexually mature, male American white pelican produces during the mating season a semilunar, horny excrescence on the bill. This "horn" is lost at the end of the mating period. On the basis of its morphology, insolubility, and indigestibility, pelican excrescence is a keratin. In appearance, the tissue is similar to horn and nail keratin, but amino acid analyses (Column 9 of Table I) show quite definitely that pelican excrescence is a pseudo- and not a eukeratin.

DISCUSSION

Chemical investigations have indicated that the insoluble, enzyme-resistant keratins, almost always of ectodermal origin, may be divided into two groups which have been called eukeratins and pseudokeratins. Proteins which have been classified as eukeratins are human hair (30), chimpanzee hair (31), cow hair (31), goat hair (32), camel hair (32), goose feathers (20), hen feathers (32), finger nails (33), cattle horn (33), rhinoceros horn (32), spiny echidna quills (34), porcupine quills (34), snake skin (20, 32), and hen's egg-shell membrane (35). Proteins which may be called pseudokeratins are human skin (25), neurokeratins (5), whale baleen (5), horse burrs (5), fish egg casing (36), spongin, probably gorgonin, and possibly turtle scutes.

It is of interest to speculate on the possible relationship of the eukeratins and pseudokeratins. Experiments involving fractional enzymatic digestion of pseudokeratins (whale baleen, human skin, horse burrs, etc.) indicate that eukeratins do not arise from pseudo-

keratins by any simple autolytic removal of the more easily digestible proteins, but that the formation of eukeratins is the result of a true synthetic process. It may be that stimulation by the corium of ectodermal cells, which normally produce pseudokeratin, results in the formation of a tissue composed primarily of eukeratins. Thus the pseudokeratins may be more closely related to the primordial ectoderm. The eukeratins, which are characterized especially by the constant ratio of their basic amino acids, may have been evolved from the ectoderm for the purpose of affording the animal extra protection against the environment.

SUMMARY

1. Gorgonin, spongin, turtle scutes, and a horny excrescence on the bill of the pelican were analyzed for nitrogen, sulfur, iodine, histidine, lysine, arginine, tyrosine, tryptophane, phenylalanine, and glycine.

2. These proteins, which may be classified as pseudokeratins, are characterized by their insolubility, resistance towards enzymatic digestion, and by their content of lysine and arginine. The latter are in the molecular ratio of approximately 4:6.

3. Specific differences in amino acid composition are also apparent. Thus gorgonin and turtle scutes contain more than 13 per cent of tyrosine, while spongin yields less than 1 per cent of this amino acid. More than 8 per cent of cystine was found in the former proteins, while spongin and pelican excrescence contain approximately 3 and 4 per cent respectively. In contrast to other pseudokeratins, turtle scutes yield more histidine and less lysine. Tryptophane was not found in either spongin or gorgonin.

4. The origin and possible interrelationship of the eukeratins and pseudokeratins are discussed.

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THE EFFECT OF TITANIUM ON THE OXIDATION OF SULFHYDRYL GROUPS BY VARIOUS TISSUES

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Small amounts of titanium are found in plant and animal tissues. According to Bertrand and Voronca-Spirt (1) the liver in animals and the polishings of cereals contain the most. More recently, Vinogradov (2) has estimated the titanium content of tissues. According to Mellor (3) it is more abundant in the earth's crust than any of the common metals except iron. Richet, Gardner, and Goodbody (4) and Pick (5) have fed animals with large amounts of titanium and found it to have little or no toxicity. There remains, however, the possibility that the titanium which is always present in animal tissues, particularly the liver, may have a metabolic function, and the following experiments were carried out on tissue suspensions to determine whether this was the case.

EXPERIMENTAL

It was necessary first to obtain a preparation of titanium that was soluble in the presence of phosphate buffer, for addition of the insoluble TiO_2 or titanous phosphate to tissue was without effect. Titanous sulfate was added to 0.05 M phosphate buffer of pH 6.7 so that the final concentration was 5.0 mg. per cc. and a precipitate of titanous phosphate formed immediately. To this 2 drops of 30 per cent H_2O_2 were added and after the mixture had stood for a few hours at room temperature the precipitate dissolved and a clear light yellow solution was obtained. This was then boiled to get rid of the excess H_2O_2 and allowed to stand for several days. Any small amount of H_2O_2 that still remained was immediately decomposed when added to the tissue by the catalase present in it. 0.05 cc. of this solution, containing 6 micrograms

of titanium as sodium pertitanate, was used in the following experiments. The solution of this salt in phosphate buffer is stable at room temperature for several weeks.

The tissues of the rat were prepared by chopping with scissors, grinding with sand after adding 0.05 M phosphate buffer, and squeezing through muslin. Varying amounts of the resulting suspension were added to the Warburg vessels and buffer added so that the final volume was 2.0 cc. When 6 micrograms of titanium were added to such suspensions, a marked inhibition

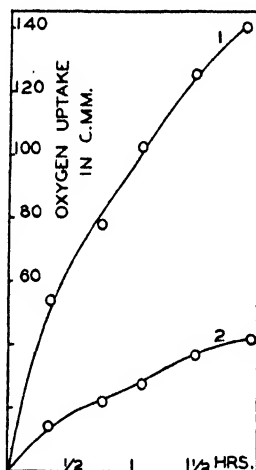


FIG. 1. The oxygen uptake of untreated rat liver suspension with and without 6 micrograms of titanium as sodium pertitanate at pH 6.7 and 37°. Curve 1, control; Curve 2, with titanium.

of the oxygen uptake occurred (Fig. 1). That this was due to the titanium and not to any traces of H_2O_2 that may have been added with it was shown by the fact that additions of relatively large amounts of H_2O_2 had no effect on the subsequent oxygen uptake of the tissue. The inhibition of the oxygen uptake of liver was the greatest, the kidney next, and the brain showed the least. The liver was therefore used in the following experiments.

In order to see whether a specific oxidation was being inhibited by the titanium, a liver suspension was made by preparing 3 gm.

of liver and centrifuging twice with 50 cc. of water containing 10 cc. of phosphate buffer of pH 6.7. After the second washing the insoluble protein that remained and which was now practically free of hemoglobin and oxidizable substrates was suspended in 10 cc. of buffer of pH 6.7 and 0.4 to 0.6 cc. used in each Warburg vessel. The titanium effect on this washed protein is negligible. Various substrates were then added to the protein with and without titanium. These included amines, amino acids, alcohols, aldehydes, hypoxanthine, glucose, lactate, succinate, citrate, acetate, choline, and cysteine. Only the oxidation of cysteine was inhibited by the presence of titanium.

It is well known that cysteine is oxidized to cystine and that certain metals catalyze this oxidation. It has been assumed that the addition of tissue to a solution of cysteine will increase the rate of this oxidation because of the iron and copper present in the tissue. It was immediately obvious, however, that the addition of washed liver suspension did more than this, because the extra oxygen uptake caused by the cysteine was much greater than could be accounted for by its oxidation to cystine. There were two possibilities to account for this. If cystine were formed from cysteine in the presence of liver, the cystine might be reduced back again by the liver protein. Such a reversible process would account for the extra oxygen uptake and the uptake would depend on the amount of protein present. But this cannot be the case, because added cystine has no effect on the uptake and because the uptake is dependent only on the concentration of cysteine and is independent within the limits of the protein concentration. Furthermore, titanium, carefully freed of excess H_2O_2 , when added to a solution of cysteine without liver actually accelerates the oxidation of the cysteine to cystine. Therefore the extra oxygen uptake of cysteine in the presence of liver protein is probably caused by a further oxidation of the cysteine itself. As no appreciable deamination occurs, this oxidation probably takes place on the sulfur.

It therefore seems probable that when cysteine is added to the liver preparation part of it is oxidized to cystine but no further and this oxidation is not inhibited by titanium. Another part of the cysteine is oxidized in a different way, taking up more oxygen, and this oxidation is inhibited by titanium. At pH

6.7 in phosphate buffer the oxidation of cysteine to cystine proceeds slowly. In the presence of liver protein the oxidation is rapid. It therefore seemed possible to arrange the conditions so that only the minimal amount of cysteine went to cystine and the amount of oxygen taken up by the rapid oxidation could be accurately measured. Such conditions were obtained when 0.4 to 0.6 cc. of liver protein was mixed with 0.25 mg. of cysteine hydrochloride (neutralized), and the results are shown in Fig. 2.

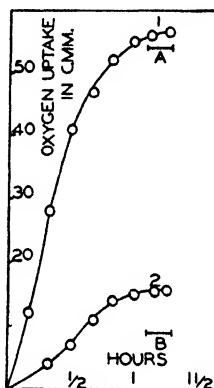


FIG. 2. The oxidation of 0.25 mg. of cysteine hydrochloride (neutralized) with 0.4 cc. of washed liver protein at pH 6.7 and 37°. Curve 1, the oxygen uptake of liver and cysteine hydrochloride from which is subtracted the uptake of the liver alone; Curve 2, the oxygen uptake of liver, cysteine hydrochloride, and 6 micrograms of titanium as sodium pertitanate from which is subtracted the uptake of the liver and titanium alone. Horizontal Line A, theoretical uptake for 3 atoms of oxygen per molecule of cysteine hydrochloride; horizontal Line B, theoretical uptake for the oxidation of cysteine hydrochloride to cystine.

If this amount of cysteine hydrochloride was oxidized to cystine, 9 c.mm. of oxygen would be taken up. Instead, 54 c.mm. are taken up and this means that 3 atoms of oxygen are used per molecule of cysteine hydrochloride, which would correspond to the production of cysteic acid. No CO_2 is given off during the oxidation. In the presence of 6 micrograms of titanium the oxidation to cysteic acid is almost completely inhibited and the uptake due to cysteine is only slightly more than the theoretical for the formation of cystine.

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As there is a 6-fold difference in the oxygen uptake of cysteine, depending on whether it is oxidized to cystine or cysteic acid, it is possible to determine from the final oxygen uptake which of the two oxidations predominates under any given set of conditions. The percentage inhibition by titanium will also be an indication, for if much of the cysteine goes to cystine the inhibition will be less. This can be shown by varying the relative concentrations of liver protein and cysteine (Table I). If too little protein is present to cause the rapid oxidation of cysteine

TABLE I

Effect of 6 Micrograms of Titanium As Sodium Pertitanate on Oxygen Uptake of 0.25 Mg. of Cysteine Hydrochloride with Varying Quantities of Washed Rat Liver Protein at pH 6.7 and 37°

The theoretical for the uptake of 3 atoms of oxygen per molecule of cysteine hydrochloride is 54 c.mm.

Time	0.2 cc. liver + cysteine hydrochloride		0.4 cc. liver + cysteine hydrochloride		0.8 cc. liver + cysteine hydrochloride	
	Alone	Titanium	Alone	Titanium	Alone	Titanium
min.	c.mm. O ₂	c.mm. O ₂	c.mm. O ₂	c.mm. O ₂	c.mm. O ₂	c.mm. O ₂
10	1	2	8	0	33	9
20	3	5	20	3	54	14
30	12	9	27	4	60	21
40	19	15	33	8	59	25
50	23	16	37	7	61	24
60	25	18	40	10	62	23
70	28	23	46	14	61	24
80	32	26	50	13	63	24
90	33	29	52	14	60	24

to the product with 3 atoms of oxygen, then a fair proportion of the cysteine goes to cystine; the final uptake is less than the theoretical for 3 atoms and the titanium inhibition is small. If the amount of liver protein is increased and the cysteine concentration is kept constant, the rate of oxidation increases but the final uptake remains the same and the percentage inhibition by titanium remains about maximal.

The catalyst responsible for the uptake of 3 atoms of oxygen is thermolabile. Boiling the liver protein for 5 minutes completely abolishes this oxidation and the uptake in the presence of the

boiled protein corresponds to the oxidation of cysteine to cystine. Titanium slightly increases the rate of this oxidation. Hemoglobin alone does not cause the uptake of 3 atoms of oxygen, so traces of this pigment which may still be present in the liver protein do not enter into the reaction. Cyanide inhibits the uptake of 3 atoms of oxygen as well as the oxidation to cystine. The inhibition by titanium decreases with increasing pH. At pH 6.4 the uptake of the 3 atoms of oxygen is slower than at pH 6.7 but the titanium inhibition is the same.

Because pertitanic acid is an oxidizing agent, it was possible, despite the fact that so little was present, that it was donating oxygen to the cysteine and the inhibition in the oxygen uptake was only apparent. To prove that this was not the case the nitroprusside test for $-SH$ groups was carried out. After the oxidation of cysteine with and without titanium had proceeded for 20 minutes, an aliquot was removed from the vessels and tested in the usual way. The nitroprusside test was almost negative in the control in which oxidation was rapid but was still present in the titanium-inhibited system. The intensity of color in this latter was about the same as in the aliquot taken from the vessel in which cysteine without liver was being oxidized. This test showed even more striking differences in the case of thioglycolic acid described below.

The evidence thus far indicates that cysteine may be oxidized by a thermolabile catalyst in liver, causing the uptake of 3 atoms of oxygen with the probable formation of the sulfonic acid. Thioglycolic acid was therefore tried to determine whether the oxidation of the sulfur atom was a general one. The results are shown in Fig. 3. In this case also a rapid oxidation without decarboxylation occurs in the presence of washed liver protein with the uptake of 3 atoms of oxygen per molecule, and it is completely inhibited by 6 micrograms of titanium. The uptake is a function of the amount of thioglycolic acid added and is independent within limits of the amount of protein present. As shown in Fig. 3, the titanium inhibition decreases after about 2 hours and the decrease is greatest where the greatest amount of thioglycolic acid is being oxidized. This shows that the inhibition is reversible. Thioglycolic acid alone in buffer of pH 6.7 is oxidized very slowly and titanium has no effect on this oxidation. When

the oxidation by liver was complete, samples were removed from the control and from the titanium-inhibited system. The nitroprusside test showed the complete absence of —SH groups in the control and a concentration of —SH groups in the titanium-

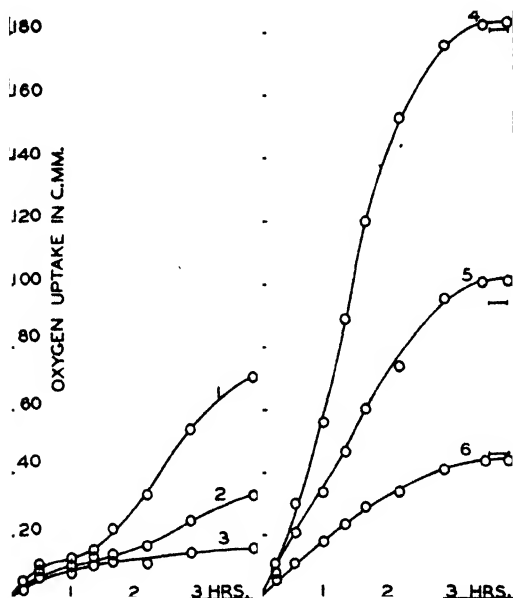


FIG. 3. The oxidation of different amounts of thioglycolic acid (neutralized) with 0.4 cc. of washed liver protein at pH 6.7 and 37° . The curves represent the oxygen uptake of the liver and the thioglycolic acid, and the liver, thioglycolic acid, and 6 micrograms of titanium, as sodium pertitanate, from which the oxygen uptake of the respective controls has been subtracted. Curve 1, liver, 0.5 mg. of thioglycolic acid, and titanium; Curve 2, liver, 0.25 mg. of thioglycolic acid, and titanium; Curve 3, liver, 0.125 mg. of thioglycolic acid, and titanium; Curve 4, liver and 0.5 mg. of thioglycolic acid; Curve 5, liver and 0.25 mg. of thioglycolic acid; Curve 6, liver and 0.125 mg. of thioglycolic acid. The horizontal lines represent the theoretical for the uptake of 3 atoms of oxygen per molecule of thioglycolic acid.

inhibited sample equal to that of the original thioglycolic acid added. This shows the complete removal of —SH groups when the oxidation was finished. This fact, with the quantitative uptake of 3 atoms of oxygen, suggests the formation of the sul-

fonic acid. The oxidation of thioglycolic acid resembles that of cysteine except that longer boiling is required completely to inhibit the oxidation of the former.

A strong acid is formed during the oxidation of these compounds, as is shown in the following experiment. The oxidation of 0.25 mg. of thioglycolic acid was allowed to proceed in the absence of buffer and the pH determined by brom-thymol blue when the oxidation was complete. The solution containing the oxidized thioglycolic acid was 0.3 pH more acid than the control, but in the presence of titanium the pH was the same as in the control.

It is interesting that glutathione is not oxidized with the uptake of 3 atoms of oxygen. When it is added to tissue, a small extra oxygen uptake occurs which can be accounted for by its oxidation to the disulfide compound. This is not inhibited by titanium. Ethyl mercaptan is only very slowly oxidized by the washed liver preparation and no definite end-points could be obtained. In this case, also, titanium inhibits the reaction.

DISCUSSION

The oxidation of cysteine to cysteic acid in the animal body has been postulated because it is believed to be a precursor of taurine. Schmidt and Clark (6) in feeding cysteic acid to dogs found evidence that it was deaminated but not further attacked. The evidence presented here indicates that cysteic acid can be formed from cysteine, but under the conditions of our experiments, deamination does not occur. Positive identification of the cysteic acid is difficult because of the lack of characteristic reactions for sulfonic acids. The oxygen uptake, however, corresponds exactly and a compound is formed which is a strong acid and which is not readily reduced to give a positive nitroprusside reaction. Since titanium is found in the livers of all animals, it is possible that it may play a rôle in regulating the oxidation of certain —SH compounds to sulfonic acids.

SUMMARY

1. 6 micrograms of titanium as sodium pertitanate inhibit the oxygen uptake of rat tissue suspensions. The liver is inhibited most, the kidney next, and the brain least.

2. If cysteine is mixed with washed liver protein at pH 6.7 in the correct proportions, 3 atoms of oxygen are taken up per molecule of cysteine, corresponding to the formation of cysteic acid.

3. This oxidation is catalyzed by a thermolabile catalyst and is inhibited completely by the sodium pertitanate. The latter has no inhibiting effect on the oxidation of cysteine to cystine, which occurs without addition of tissue.

4. Thioglycolic acid is oxidized by the same protein with the uptake of 3 atoms of oxygen per molecule and this oxidation is also inhibited by sodium pertitanate.

5. The nitroprusside test is negative at the end of the oxidation of both substances but is still as positive when sodium pertitanate is present as in the controls without liver protein.

6. Glutathione is not oxidized in this way by the liver protein. It is oxidized to the disulfide and this is not inhibited by the pertitanate. Ethyl mercaptan is oxidized slowly by the liver protein and the pertitanate inhibits the reaction.

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SOLUBILITY OF BONE SALT

IV. SOLUBILITY OF BONE IN BIOLOGICAL FLUIDS

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When bone or similar artificially prepared salts are equilibrated with inorganic solutions, the value for $p[\text{Ca}^{++}]^2 \times [\text{PO}_4^{=}]^2$ obtained at equilibrium increases as the amount of solid is increased (1).¹ The value for $p[\text{Ca}^{++}]^2[\text{PO}_4^{=}]^2$ obtained with a minimum amount of solid amounted to 23.1 ± 0.3 . This corresponds essentially to that calculated for normal blood plasma from the $[\text{Ca}^{++}]$ (as determined by means of the frog heart), the inorganic phosphate concentration, and the pH (2).

Because this value ($p\ 23.1 \pm 0.3$) represents the concentration of calcium and phosphate at which precipitation can be initiated from salt solutions, it was considered advisable to determine whether this was also true for biological solutions. The results could be expected to show by direct experiment whether the bone salt could be formed spontaneously in biological fluids. This paper deals with the results of experiments on the equilibration of bone with blood plasma and ascitic fluid. They confirm the results obtained on pure solutions and indicate that the blood plasma is not supersaturated in respect to the first solid formed upon the precipitation of the bone salt.

Secondarily, the equilibration of blood plasma with large amounts of bone powder might be expected to indicate more clearly the actual conditions necessary for the solution of the bone salt. This is so for the following reasons. The conditions

¹ Ions enclosed in brackets refer to moles per liter of solution. The term $p[\text{Ca}^{++}]^2[\text{PO}_4^{=}]^2$ represents the common logarithm of the reciprocal of the ion product $[\text{Ca}^{++}]^2 \times [\text{PO}_4^{=}]^2$. Therefore, decreases in $[\text{Ca}^{++}]^2 \times [\text{PO}_4^{=}]^2$ are represented logarithmically by increases in $p[\text{Ca}^{++}]^2[\text{PO}_4^{=}]^2$.

necessary for the solution of the solid are not adequately defined by the expression $[Ca^{++}]^3 \times [PO_4^{=}]^2$. The expression serves only as a convenient means of comparison with previous work in which it was considered valid. In order that a better expression may be evolved, accumulation of data concerning the composition of fluids in equilibrium with large amounts of solids of differing compositions appears desirable. It is difficult to prepare artificial precipitates with carbonate contents as high as that in bone under conditions which preclude the probability that calcium carbonate will be precipitated as such. For the same reason, equilibration with inorganic solutions having the same ionic concentration as blood plasma is open to objection. Therefore, apart from the possible biological significance of the results, equilibration of bone with blood plasma affords conditions which are otherwise difficult to reproduce artificially.

The results show that $p[Ca^{++}]^3 \times [PO_4^{=}]^2$ increased as the amount of bone or glycerol ash of bone² increased. With artificial precipitates, the maximum value for $p[Ca^{++}]^3 \times [PO_4^{=}]^2$ was apparently reached at about 150 mg. of solid per liter. With bone, the value continued to increase gradually when the amount was increased to such an extent (more than 10 gm. per liter) that the organic matter of the bone introduced a disturbing factor. The equilibrations with the glycerol ash of bone are free from this objection and the concentrations of the ions in equilibrium with the largest amounts probably most closely approach the concentrations at which bone dissolves.

EXPERIMENTAL

To 3 liter quantities of sterile horse serum was added 0.1 per cent of thymol and the solution was incubated at 37° until the inorganic phosphate had increased to a maximum constant value. The pH of the serum was adjusted to its original value with sterile NaOH during the course of the incubation. It was found

² Glycerol ash of bone is bone powder from which the organic matter has been removed by heating with glycerol and KOH. The residue is subsequently washed free of glycerol and KOH with water. The process has been claimed (3) to leave the inorganic portion unchanged. In our hands, comparison of the ratio of phosphate to carbon dioxide in the fresh bone and residue indicated a loss of 5 to 10 per cent of the carbon dioxide.

that the pH and inorganic phosphate of the serum would remain essentially constant during the subsequent equilibration if the incubation was continued for 2 to 3 weeks.³ Na_2HPO_4 was added in two of the experiments (Tables I and II) to decrease the $p[\text{Ca}^{++}][\text{PO}_4]^{1/2}$ of the serum below 23.5. Human blood serum and ascitic fluid were similarly prepared. Sterile flasks of 200 to 300 cc. capacity were filled with the serum. To these flasks were added the amounts of bone powder or glycerol ash recorded on Fig. 1 and in Tables I and II. The solutions were stoppered and rotated at 37° for 5 days. Any flasks showing evidence of bacterial contamination were discarded. Samples of the serum

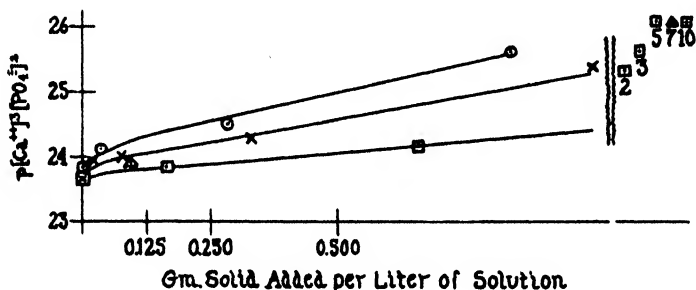


FIG. 1. Ion product in horse serum. \circ and \times represent bone from beef; \square , bone from old horse; \triangle , glycerol ash of bone from old horse. The points plotted after the break in the chart give the ion products when the amount of saturating body was large. The figures accompanying the points designate the gm. of solid per liter of solution.

were centrifuged under oil at 3400 R.P.M. for 5 minutes and determinations of H^+ , total CO_2 , Ca, and inorganic phosphate content were made without delay.

The bone powder was cut from bone cortex by means of a motor-driven, steel milling head. That portion which passed a 100 mesh sieve was washed with water,⁴ alcohol, and ether,

³ The Folin-Wu blood sugar reducing value essentially disappears from horse serum during this time. Part of the phospholipid settles out together with a small amount of protein.

⁴ Fetal bone was successively suspended in water and squeezed in a press several times to remove, as far as possible, soluble protein material.

TABLE I

$p[Ca^{++}]^3[PO_4^{==}]^2$ Found by Equilibrating Fetal and Adult Beef Bone and the Glycerol Ash of Each in Horse Blood Serum

Composition of the blood serum after equilibration for 5 days. Total protein = 5.48 per cent.

Solid added		Total Ca	Inorganic P	pH	CO ₂	$p[Ca^{++}]^3[PO_4^{==}]^2$
	mg. per l.	mm per l.	mm per l.		mm per l.	
	0	2.10	1.69	7.39	17.1	23.42
F.	1	2.26	1.70	7.38	17.2	23.36
A.	1	2.0	1.68	7.38	17.4	23.50
F.	183	1.61	1.47	7.36	17.1	23.95
"	710	1.66	1.32	6.76	17.2	25.54
A.	710	1.15	1.08	7.29	16.6	24.83
F.	2,220	1.56	1.20	6.78	16.8	25.69
A.	2,220	0.80	0.84	7.27	16.7	25.59
F.	11,100	1.41	1.18	6.74	16.0	25.92
A.	11,100	0.70	0.84	7.24	15.9	25.84
Ga. of F.	5,550	0.60	0.56	6.76	16.0	27.66
" " A.	5,550	0.5	0.58	6.74	15.4	26.88
F.	52,000	2.55	1.15	6.65	15.0	25.31

F. = fetal bone. A. = adult bone. Ga. = glycerol ash.

TABLE II

$p[Ca^{++}]^3[PO_4^{==}]^2$ Found by Equilibrating Human Bone and Its Glycerol Ash in Human Ascitic Fluid

Composition of ascitic fluid after equilibration for 5 days. Total protein = 0.51 per cent.

Solid added		Total Ca	Inorganic P	pH	CO ₂	$p[Ca^{++}]^3[PO_4^{==}]^2$
	mg. per l.	mm per l.	mm per l.		mm per l.	
	0	1.15	1.84	7.38	19.8	23.28
A.	1	1.21	1.82	7.42	19.8	23.19
Ga. of A.	1	1.02	1.84	7.42	19.9	23.42
A.	150	0.78	1.61	7.35	19.8	24.07
Ga. of A.	83	0.95	1.70	7.38	19.6	23.69
A.	660	0.46	1.37	7.28	19.8	25.02
"	2,000	0.32	1.13	7.27	19.5	25.67
Ga. of A.	1,000	0.57	1.24	7.27	19.9	24.92
A.	7,500	0.32	0.86	7.22	19.5	26.02
"	22,000	0.65	0.77	7.02	19.1	25.67
Ga. of A.	13,300	0.35	0.85	7.27	18.3	25.92
A.	44,000	0.79	0.74	7.14	18.1	25.11

A. = adult bone. Ga. = glycerol ash.

and dried at 100°. Glycerol ash of bone was prepared according to the procedure of Gabriel (3).

Calcium was determined by the method of Fiske and Logan (4), phosphate by the Fiske and Subbarow procedure (5), and CO₂ by the manometric Van Slyke and Neill procedure (6). [Ca⁺⁺] of blood plasma and ascitic fluid was calculated from the total Ca and the protein content as described by McLean and Hastings (7). Calculation of the $p[Ca^{++}]^3[PO_4^{=}]^2$ was carried out as previously described (1).

Results

The results of three equilibrations are shown on Fig. 1, and of two others in Tables I and II.

The increase in $p[Ca^{++}]^3 \times [PO_4^{=}]^2$ was less with a given increase in old bone as compared to young bone, but the difference is not great, and may not be due entirely to variations in the composition of the inorganic portion of the solid phase. For instance, the crystals in the bone particles are embedded in solid protein. That portion lying in the freshly cut surfaces of the particles can undoubtedly come in contact with the solution. It has not been determined whether or not compact old bone and less densely calcified young bone may differ in respect to the proportion of the remaining crystals that can be considered as being in contact with the solution.

During equilibration, part of the organic portion of the bone goes into colloidal solution and may materially influence the results. After equilibration with 50 gm. of fetal bone per liter, the effect is obvious because the total calcium concentration of the equilibrated serum was higher than that of the original serum (Tables I and II). The differences between the maximum value of $p[Ca^{++}]^3 \times [PO_4^{=}]^2$ found by us and others on bone (2) (p 25.5 to 26.0) and that found for artificial precipitates (1) (p 26.5 to 27.5) are probably, at least in part, caused by the interference of organic material of the bone. It should be noted that the maximum values obtained for the ion product of the glycerol ash (Table I) (p 26.88, 27.66) are similar to those found for artificial precipitates.

SUMMARY

The calcium and inorganic phosphate concentrations of blood serum or ascitic fluid are not decreased in detectable amounts in contact with a small amount of bone (1 mg. per liter). As the amount of solid is increased, the concentrations of these constituents are decreased.

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THE EARLY EFFECTS OF PARATHYROID HORMONE ON THE BLOOD AND URINE

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Following the administration of the parathyroid hormone to young dogs, the urinary phosphate excretion increases, the blood inorganic phosphate usually decreases, and the plasma calcium increases (1-5). The increase of phosphate excretion in the urine occurs in advance of the maximum blood calcium increase. These experimental findings have been interpreted to indicate that the solution of bone is caused by depletion of blood and tissue inorganic phosphate resulting from increased excretion by the kidneys.

Histological changes in the bone have been observed very soon after administration of the hormone, indicating that stimulation of cellular activity in the tissue itself caused solution of the bone (6).

Previous experiments which included determinations of the blood levels of calcium and inorganic phosphate and also the urinary excretion of these substances have been carried out for 1 (1, 2) and 3 day periods (3-5, 7, 8). The results have clearly shown the influence of the hormone on calcium metabolism during extended experimental periods but the intimate details of the immediate changes have not been so well established. The effects of the hormone on the blood concentration of calcium and phosphate have been observed for shorter periods of time (9).

The hormone has also been administered to nephrectomized dogs (10). The results indicate that large doses produce no greater increase of blood calcium than that observed as a result of nephrectomy alone. The phosphate retention resulting from

nephrectomy increased the blood inorganic phosphate 80 per cent in 2 to 6 hours. (This increase of blood phosphate is greater than we have observed in the terminal stages resulting from the administration of fatal doses of the hormone.) As a result of the rapid accumulation of phosphate in the blood and tissues following nephrectomy, the solution of bone would be difficult to demonstrate. The results, therefore, would not appear to be conclusive evidence in support of the thesis that the action of the hormone is exclusively on the kidney.

The experiments reported here were designed to answer the following questions: (1) are the changes resulting from administration of the hormone entirely due to the increased excretion of phosphate by the kidneys, or (2) do the chemical changes indicate that the hormone also stimulates active solution of bone by the bone cells?

For that purpose, the urinary excretion and blood levels of calcium, magnesium, and phosphate were compared and the excretion of bases, chloride, and nitrogenous substances was examined at 1 hour intervals for 4 hours after administration of single, fairly large doses of parathyroid hormone to fasting young dogs. (Subsequent observations were made at longer intervals of time.) The time interval chosen is much shorter than has been previously employed and is near the shortest at which a definite response in both blood and urine can be observed.

The results indicate that the solution of the bone occurs almost immediately, because a definite rise of blood calcium occurs during the 1st hour. Increased excretion of phosphate by the kidneys is evident during the 1st hour in four of the experiments, and was accompanied by a decrease in the plasma inorganic phosphate. The increase of phosphate excretion in the 1st hour was, however, not more than twice the hourly fluctuation observed in normal fasting animals. That active solution of bone is also stimulated seems likely from the fact that the rise of blood calcium during the 1st hour was twice as great when the plasma phosphate decreased very little or not at all. This interpretation likewise appears to be favored by the reaction observed after 12 to 24 hours. At this time, the plasma inorganic phosphate is increased owing to retention by the kidneys, yet the blood calcium continues to remain above the normal level.

EXPERIMENTAL

Five young dogs were fasted 3 to 5 days. To each of three of them, the amount of parathyroid hormone shown on Figs. 1 to 3 was administered subcutaneously. To each of the other two, a similar dose was administered intravenously (Figs. 4 to 5). The dogs were catheterized each hour. The bladder was washed out with distilled water. The phosphate was determined in the washings and in the undiluted urine. The hourly volume

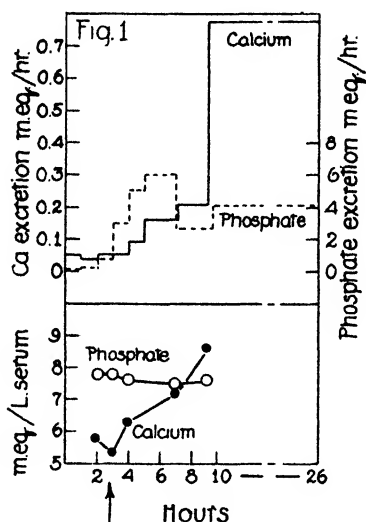


FIG. 1. Calcium and inorganic phosphate concentration of the blood and excretion in the urine following subcutaneous administration (indicated by the arrow) of 6.6 units (U.S.P. XI) of parathyroid hormone.

of urine was calculated from the volume of undiluted urine obtained and the amount of phosphate in the washings. The dogs were allowed to rest in a metabolism cage following each catheterization.

Total nitrogen was determined by the Kjeldahl procedure (11), inorganic phosphate by the method of Fiske and Subbarow (12), and calcium by procedures previously described (13). Total base was determined by the procedure of Fiske (14).

Results

The results of the experiments are shown in Figs. 1 to 5. Before administration of the parathyroid hormone, the urinary phosphate excretion of the fasting young dogs was not constant from hour to hour. Analyses of specimens from the same animal on suc-

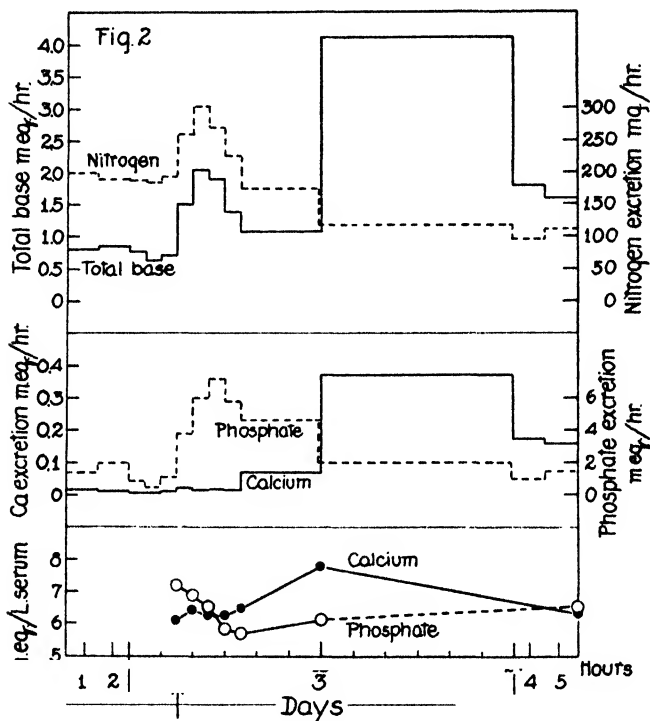


FIG. 2. Calcium and inorganic phosphate concentration of the blood and excretion in the urine following subcutaneous administration (indicated by the arrow) of 6.7 units of parathyroid hormone.

cessive days indicated that it was not possible to predict at what time the phosphate excretion would be increasing or decreasing. Consequently, the parathyroid hormone was administered to different dogs when the excretion was falling and when it was rising.

In the 1st hour after the subcutaneous administration of the

hormons, the phosphate of the urine of the three dogs (Figs. 1 to 3) increased 2.3, 2.6, and 1.1 milliequivalents as compared with the previous hour. The inorganic phosphate of the plasma

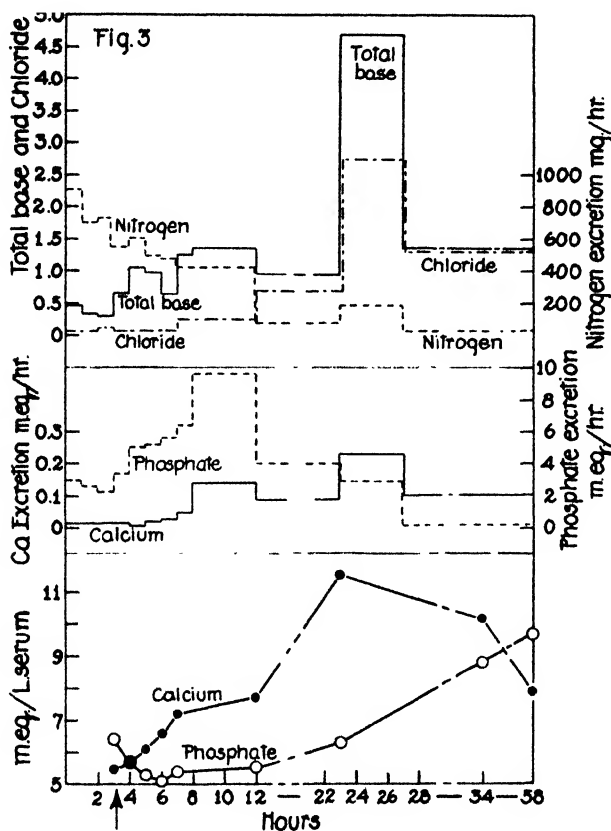


FIG. 3. Calcium and inorganic phosphate concentration of the blood and excretion in the urine following subcutaneous administration (indicated by the arrow) of 34.0 units of parathyroid hormone.

dropped in this time 0.17, 0.3, and 0.7 milliequivalent per liter respectively. In the 1st hour after the intravenous administration of a large amount of the hormone (34 units¹ per kilo, Fig. 5),

¹ The unit is defined by the United States Pharmacopoeia XI.

similar changes occurred. With a smaller dose intravenously (8.3 units¹ per kilo, Fig. 4), increase of phosphate excretion and decrease of plasma inorganic phosphate did not occur until the 2nd hour.

The plasma calcium increased 0.3 milliequivalent per liter or more in all experiments during the 1st hour. Dog 4, in which no decrease of plasma phosphate occurred during the 1st hour, and Dog 1, in which the decrease was slight (0.17 milliequivalent per liter), showed the largest increase of plasma calcium (0.6

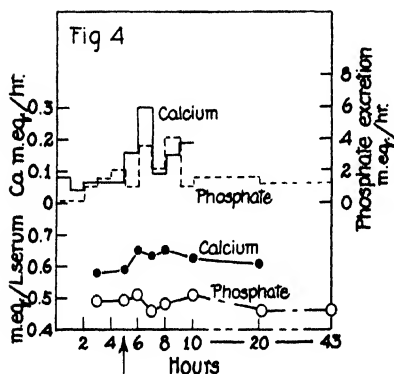


FIG. 4. Calcium and inorganic phosphate concentration of the blood and excretion in the urine following intravenous administration (indicated by the arrow) of 8.3 units of parathyroid hormone.

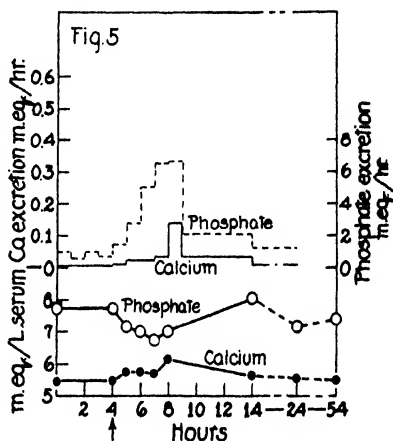


FIG. 5. Calcium and inorganic phosphate concentration of the blood and excretion in the urine following intravenous administration (indicated by the arrow) of 34.0 units of parathyroid hormone.

and 1 milliequivalent respectively). Urine calcium increased during the 1st hour in two experiments. Definite increases occurred later in all experiments.

The changes observed in the 2nd to the 4th hour indicated an extension of the changes observed in the 1st hour; namely, the phosphate and calcium excretion continued to increase, the plasma phosphate now decreased in all experiments, and the plasma calcium continued to increase. The increase of plasma phosphate, which occurred 18 to 24 hours after administration

of the hormone, is apparently due to retention by the kidneys, because, at that time, the excretion of nitrogen was decreased. In the case of Dog 3, the nitrogen excretion after 18 hours decreased to less than 10 per cent of the level which prevailed before the administration.

Relation of Phosphate to Nitrogen Excretion—It appears evident that the increased excretion of phosphate in the urine was not accompanied by an increase of nitrogen excretion such as occurs with increased catabolism of soft tissues. In Table I are given the 24 hour urinary excretions of phosphate and nitrogen by Dog 2. These results coincide with similar observations previously made during 3 day metabolism periods (5).

TABLE I
*Nitrogen and Phosphate Excretion after Parathyroid
Hormone Administration*

Day of fast	Urine total N	Inorganic P	Ratio N:P
	mg.	mg.	
3rd	4820	374	12.9
4th	4584	492	9.3
5th*	4399	1080	4.1
6th	2212	255	8.6
7th	5250	438	12.0

* 6.7 units of parathyroid hormone per kilo were administered subcutaneously at the beginning of the 5th day of the fast.

The results of comparison of the N:P ratio of the urine for shorter periods are of less certain value. This is because the normal hourly phosphate excretion is variable, and because retention of nitrogen evidently occurred after 12 to 24 hours. Dog 3, which received the largest dose of hormone subcutaneously (34 units per kilo), showed no increase of urinary N excretion for 5 hours after administration, during which time the phosphate excretion had increased from 2.3 to 6.4 milliequivalents per hour. During the next 16 hours, the average nitrogen excretion was slightly lower, while the average phosphate excretion had increased to 9.8 milliequivalents per hour. Analysis of the urine for the succeeding hours indicated a retention of both nitrogen and phosphate. The nitrogen excretion of Dog 1 increased for

5 hours after administration of the hormone and then decreased to about half the level of the first period, while the phosphate excretion continued for 19 hours at a level well above the average hourly excretion expected from a dog of its size.

Results Not Given in Detail—Excretion of creatine and creatinine, magnesium, and ammonia, and the pH were determined but are not given in detail because they showed no unexpected changes. It was considered possible that increased phosphate excretion might involve creatine or creatinine. The hourly excretions of creatine and creatinine, as well as the hourly volumes of urine, were essentially constant until nitrogen excretion became markedly decreased. Following the administration of the parathyroid hormone, excretion of magnesium in the urine increased (Dogs 1, 2, and 3) as did also ammonia excretion (Dog 3). The pH of the urine dropped from 6.8 to 5.8 (Dog 3). Until marked decrease of nitrogen excretion occurred, these changes approximately paralleled the total base increase. The changes are such as would be expected to accompany the increased anion (phosphate) excretion. The increase in volume of the urine, together with the increase of base and chloride excretion and the decrease in nitrogen excretion which eventually occurred after a large dose of the hormone, is apparently due to kidney damage.

SUMMARY

The urinary phosphate excretion increases and the plasma inorganic phosphate usually decreases during the 1st hour after the administration of the parathyroid hormone. The results are interpreted to indicate that active excretion of phosphate by the kidney is one of the first effects of excess of the hormone. Because the blood calcium increased in the same time, whether or not the phosphate decreased, it is reasonable to suppose that active solution of bone also took place in the 1st hour.

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THE INFLUENCE OF COCAINE FEEDING ON THE LIVER LIPIDS OF THE WHITE MOUSE*

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The prominent rôle attributed to the liver in fat metabolism has led to the study of many substances which produce fatty infiltration in this organ (see Best and associates (1)). Ehrlich (2) described an extensive fatty change in the livers of cocaine-fed mice. An experiment was devised to determine the amount and nature of these lipid changes.

Methods

Three groups of albino mice were placed on low, medium, and high daily dosages of cocaine hydrochloride solution administered *per os* by means of a blunt needle and a tuberculin syringe. The doses beginning at 0.2, 0.4, and 0.6 mg. in 0.02 cc. per day were gradually increased to 1.2, 2.4, and 4.8 mg., respectively, at the end of 60 days. The total doses given were approximately 20 mg., 40 mg., and 70 mg., respectively. The final body weights ranged from 110 to 140 gm.

The mice were killed by a blow on the head and the livers removed immediately. A small portion of each liver was reserved for fat staining by the usual method (Sudan IV).

The size of the livers (*ca.* 1 gm.) did not permit a direct determination of moisture content. Therefore, the samples to be analyzed were ground with a known weight of sand, according to the technique of Bloor (3), and extracted three times with hot alcohol-ether (3:1). The moisture content was calculated from

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the weights of the dry, fat-free residue (minus sand) plus the total lipid. The lipid analyses were made by standard procedures (4, 5).

Results

Histological—The vacuolar degeneration after cocaine feeding described by Ehrlich (2) and others (6) was observed in every liver. A variable increase in cell size (up to 2 or 3 times normal) was noted in the livers of all treated animals. The fatty infiltration (estimated histologically) was inconstant and apparently due to individual susceptibility and not to the size of the dose. For example, of the seven mice receiving the medium dosage, three showed "slight," one showed "moderate," and three showed "extensive" fatty change. Since more than half of the cocaine-fed animals showed a fatty change, the presence of fat hardly seemed accidental (which was Ehrlich's finding).

Chemical—Only a minority of the mice showed large changes in their liver lipids; these data are emphasized in Table I by bold-faced type. These animals were grouped and described as showing extensive liver damage in Table II.

Mg. of Liver per Gm. of Mouse—The treated animals averaged 21 to 24 per cent larger livers than the normals (Table II). Therefore, the enlargement, first noted by Ehrlich, is probably an indication of a general toxic reaction.

Phospholipid—The average phospholipid values, in contrast to those of the other lipids, showed a striking constancy, the value for the group showing extensive fatty change being substantially the same as that for the controls; *viz.*, 12.6 and 12.4 gm. per 100 gm.,¹ respectively. The constancy of the phospholipid content does not support the idea of Theis (7) that in liver injury phospholipid is diminished because of a failure to convert neutral fat to phospholipid. The constancy fits in with the observations of Terroine (8) and Terroine and Belin (9) who relate the *élément constant* to the phospholipid content (compare (10-13)).

¹ Since certain samples showed an enormous infiltration of neutral fat and cholesterol, in contrast to a marked constancy in the phospholipid content, it was considered advisable to calculate the data, with the exception of the moisture content, on the basis of the fat-free, dry weight. The values, expressed as gm. per 100 gm., therefore, refer to the gm. of lipid per 100 gm. of fat-free, dry tissue.

TABLE I
Data on Cocaine-Fed White Mice

	Mouse No.*	Mouse weight	Liver weight	Mg. liver per gm. mouse	Liver†				
					Moisture	Neutral fat	Phospho-lipid	Cholesterol	Total lipid
		gm.	gm.		per cent	gm. per 100 gm.	gm. per 100 gm.	gm. per 100 gm.	gm. per 100 gm.
Controls	4 N.	19.63	1.280	65.2	77.9	4.3	12.2	2.32	18.8
	5 "	27.14	1.475	54.3	76.1	4.7	12.1	1.71	18.5
	8 "	17.50	0.846	48.3	75.3	4.8	14.1	2.09	21.0
	11 "	19.07	1.108	58.1	73.8	4.4	11.4	1.76	17.6
	16 " ‡	25.32	1.420	75.4	76.6	1.8	12.6	2.00	16.4
	20 "	25.82	1.293	50.1	74.3	3.1	13.1	1.79	18.0
	C ₁ "	21.20	1.500	70.8	75.8	3.6	11.6	1.84	17.0
Low dosage	C ₂ "	19.80	1.300	65.7	73.7	5.5	11.7	2.07	19.3
	9 E.	18.43	1.209	65.6	76.9	26.6§	13.3	5.20	45.1
	12 S.	17.17	1.232	71.8	74.4	4.1	11.3	1.85	17.3
	14 M.	22.92	1.506	65.7	76.3	5.5	14.3	2.40	22.2
	17 "	18.02	1.454	80.8	77.1	7.7	10.9	3.13	21.7
	21 S.	22.47	1.438	64.0	75.7	4.1	13.6	2.35	20.1
Medium dosage	1 E.	14.60	0.717	49.1	69.2	81.0	10.4	3.88	95.3
	2 S.	23.00	1.612	70.1	76.5	3.6	10.9	1.82	16.3
	6 E.	22.51	1.546	68.7	80.6	12.1	14.9	5.08	32.1
	15 M.	19.84	1.354	68.2	76.7	5.1	11.7	2.15	19.0
	18 S.	23.06	1.654	71.7	77.1	4.9	15.0	2.08	22.0
	22 E.	24.48	2.754	112.5	74.1	83.5	12.2	6.67	102.4
High dosage	23 S.	21.42	1.507	70.4	75.6	4.3	13.0	2.47	19.8
	3 E.	13.10	0.772	58.9	77.6	17.5	11.8	3.76	33.1
	7 "	17.68	1.080	61.1	80.7	8.3	11.9	4.50	24.7
	10 "	20.18	1.836	91.0	78.9	16.7	13.7	4.75	35.2
	13 S.	15.71	1.066	67.9	75.5	4.4	11.8	1.85	18.1
	19 "	18.63	1.454	78.0	75.8	5.3	12.2	2.64	20.1
	24 M.	21.20	1.426	67.3	74.8	4.1	13.3	2.58	20.0

* E. = extensive damage; M. = moderate damage; S. = slight damage; N. = normal.

† Since certain samples contain an unusually large amount of fatty material, the data, with the exception of the moisture content, are calculated on the basis of the fat-free, dry weight of the tissue, and are expressed as gm. of lipid per 100 gm. of fat-free tissue. Such values may exceed 100.

‡ Pregnant.

§ The results of animals showing extensive damage are given in bold-faced type.

TABLE II
Average Liver Lipid Values of Cocaine-Fed Mice Grouped According to Extent of Liver Damage

The results are calculated on a fat-free, dry weight basis.

Extent of liver damage	Body weight	Mg. liver per gm. mouse	Water content*	Neutral fat	Phospholipid	Cholesterol	Total lipid	Phospholipid to cholesterol ratio	Phospholipid to neutral fat ratio†
	gm.		per cent	gm. per 100 gm.	gm. per 100 gm.	gm. per 100 gm.	gm. per 100 gm.		
Controls (8)	21.94	58.6	75.4	4.0	12.4	1.95	18.3	6.38	68:22
Slight (7)	20.21 (-7.9)†	70.5 (+20.5)	75.8 (+0.5)	4.4 (+10.0)	12.5 (+0.8)	2.15 (+10.3)	19.1 (+4.4)	5.90 (-7.5)	66:23
Moderate (4)	20.49 (-6.6)	70.5 (+20.5)	76.2 (+1.1)	5.6 (+40.0)	12.5 (+0.8)	2.57 (+31.8)	20.7 (+13.1)	5.00 (-21.6)	61:27
Extensive (7)	18.71 (-14.7)	72.4 (+23.6)	76.9 (+2.0)	35.1 (+778)	12.6 (+1.6)	4.83 (+148)	52.6 (+187)	2.67 (-58.0)	32:57

* Calculated on the moist weight of tissue.

† Calculated as the percentage of total lipid.

‡ Values in parentheses represent percentage changes.

Neutral Fat and Cholesterol—The neutral fat and cholesterol fractions increased greatly with increasing degree of liver injury. However, in extensive injury the increase of neutral fat (778 per cent) far exceeded that of cholesterol (148 per cent). The results indicate an extensive mobilization of both these lipids from the depots, as well as the possibility of a decrease in their metabolism by the liver. The decrease in the phospholipid to cholesterol ratio (Table II) accompanying increased liver damage (and hence probably decreased liver function) is of interest in view of the findings of Bloor (14) that the phospholipid to cholesterol ratio of muscles decreases with decreasing activity. The concurrent decrease in the ratio of phospholipid to neutral fat (Table II) is of especial interest, since it clearly shows that a reversal of the ratio (from normal) may result from a change in the neutral fat content only.

Total Lipid—The changes in the total lipid paralleled those of the neutral fat (15). The extraordinary degree of fatty infiltration which may occur was illustrated best in the data for Mice 1 and 22, for which the total lipid values were 95.3 and 102.4 gm. per 100 gm., respectively.

SUMMARY

1. Examination of the livers of mice fed various dosage levels of cocaine showed (a) a vacuolar degeneration in every case and (b) an extensive fatty infiltration in nearly half the cases.

2. Neutral fat and cholesterol increased greatly with the increasing degree of liver injury. In contrast, the phospholipid values showed a striking constancy.

3. The constant proportion of phospholipid to non-lipid constituents, despite an enormous infiltration of neutral fat and cholesterol, emphasizes the importance of this substance as a true cellular constituent.

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A FURTHER CONTRIBUTION ON THE RELATIONSHIP OF THE STRUCTURE OF *l*-CARNOSINE TO ITS DEPRESSOR ACTIVITY

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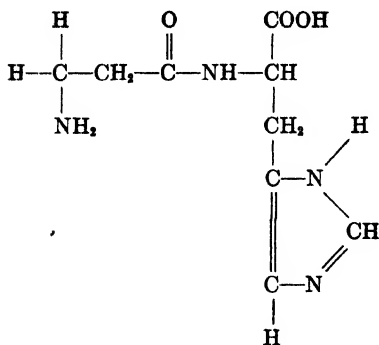
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(Received for publication, December 17, 1938)

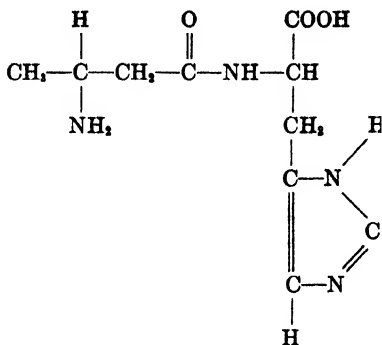
The finding that the α -alanyl peptides of *l*-histidine in contrast to carnosine, the β -alanyl peptide, possessed no appreciable depressor activity has demonstrated the importance of the position of the amino group in the alanyl moiety of carnosine for production of its pharmacodynamic effect (1). Furthermore, the lack of appreciable effect of the next higher and lower homologues of carnosine, γ -aminobutyryl-*l*-histidine and glycyl-*l*-histidine (2), on the blood pressure emphasized that the important factor with respect to the amino group is its presence in the β position rather than its attachment to the terminal carbon atom of the acyl radical of carnosine.

In order to ascertain whether other β -aminoacyl peptides of *l*-histidine possessed a carnosine-like action we have now prepared the peptides of the optical isomers of β -amino-*n*-butyric acid and β -aminoisobutyric acid with *l*-histidine. The *l*- β -amino-*n*-butyryl-*l*-histidine and its diastereoisomer were chosen because they would enable us to test whether the β -amino group must be attached to a primary carbon atom. Furthermore, we felt we could conclude that other straight chain β -aminoacyl derivatives of histidine would very likely be ineffective if both of these peptides should prove inactive with regard to depressor effect. On the other hand the *l*- β -aminoisobutyryl-*l*-histidine and its diastereoisomer were of much interest, since they still retained the amino group β to the peptide bond and attached to a primary carbon atom as in carnosine, but have 1 of the hydrogens on the α -carbon atom replaced by a methyl group. Testing these peptides would enable

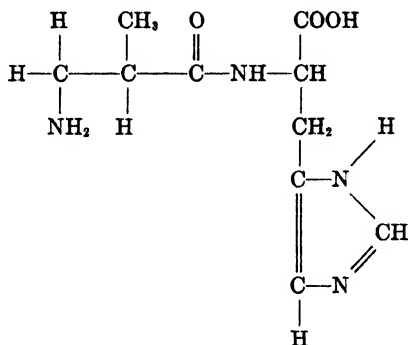
us to determine whether the presence of 2 hydrogens on the α -carbon atom in carnosine is requisite for its depressor action. The following formulas show the structural relation of these peptides to carnosine.



β -Alanyl-*l*-histidine
(carnosine)



β -Amino-*n*-butyryl-*l*-histidine



β -Aminoisobutyryl-*l*-histidine

For the synthesis of *d*- β -amino-*n*-butyryl-*l*-histidine and *l*- β -amino-*n*-butyryl-*l*-histidine, *dl*- β -amino-*n*-butyric acid was prepared by amination of crotonic acid according to the directions of Fischer and Scheibler (3). The highly insoluble carbobenzoxy derivative was employed for the isolation of the amino acid from the reaction mixture and was used directly for resolution. This was accomplished by use of both *d*- and *l*- α -phenylethylamine, as

described by Ingersoll for the resolution of various other compounds (4). This method was considerably more convenient and gave much greater yields than the method previously reported by Fischer and Scheibler in which the methyl ester was resolved with *d*-camphorsulfonic acid. The optically active peptides were prepared through the acid chlorides by procedures quite analogous to those employed for the synthesis of glycyl-*l*-histidine (2), although the acid chlorides proved to be more stable than the corresponding glycyl derivative. Both of the free peptides were isolated in crystalline form.

For the β -aminoisobutyryl peptides, *dl*- β -aminoisobutyric acid was prepared from α -bromopropionic acid. The latter was converted to α -cyanopropionic acid by treatment with NaCN and the resulting nitrile was reduced to give *dl*- β -aminoisobutyric acid. Again the carbobenzoxy derivative proved very useful for isolation of the amino acid from the reaction mixture. Resolution was accomplished by means of *d*- and *l*- α -phenylethylamine. The peptides which were obtained in the free form in crystalline condition were prepared by the same general lines of synthesis employed for the *d*(-)-alanyl-*l*(-)-histidine.

Blood pressure experiments with these peptides were carried out on cats under amytal anesthesia. In 20 times the dose of *l*-carnosine there was no appreciable effect observed from the injection of the peptides in question.

From the results of the present investigation and those previously reported by us, the following facts concerning the relation of structure to the depressor activity of carnosine have been established.

The compound must possess the correct spatial configuration, since *d*-carnosine in contrast to *l*-carnosine is inactive (5). The amino group of the acyl moiety of carnosine must be in the β position. This was evidenced by the fact that both *d*- α -alanyl-*l*-histidine and *l*- α -alanyl-*l*-histidine possessed no depressor action whatever. Furthermore, the amino group must be attached to a primary carbon atom and the hydrogen atoms attached to the α -carbon atom of the acyl moiety cannot be substituted by an alkyl group. These last two points have been demonstrated in the present report by the study of the β -amino-*n*-butyryl and the β -aminoisobutyryl derivatives of *l*-histidine.

EXPERIMENTAL

Preparation of Carbobenzoxy-dl- β -Amino-n-Butyric Acid— β -Amino-n-butyric acid was prepared according to the directions of Fischer and Scheibler (3) by the addition of ammonia to crotonic acid. After the ammonia had been removed from the reaction mixture, the solution was made just acid to Congo red with HCl and decolorized with norit. The nearly colorless solution was then made alkaline to phenolphthalein with NaOH and converted into the readily crystallizable carbobenzoxy derivative in the usual manner. The compound melted at 126°.¹ The recrystallized, air-dried material gave the following analytical values.

$C_{13}H_{15}NO_4$. Calculated, N 5.90; found, N 5.74

Resolution of Carbobenzoxy-dl- β -Amino-n-Butyric Acid—47.4 gm. of carbobenzoxy-dl- β -amino-n-butyric acid were dissolved in 600 cc. of hot ethyl acetate and 24.2 gm. of *l*- α -phenylethylamine were added. The solution was cooled for 24 hours with occasional stirring. The salt which crystallized from solution was removed by filtration and washed with cold ethyl acetate. It melted at 100°. Three recrystallizations from ethyl acetate raised the melting point to 114°. The yield of the *l*- α -phenylethylamine salt of carbobenzoxy-d- β -amino-n-butyric acid was 18 gm. The compound had the following analytical values.

$C_{20}H_{25}N_2O_4$. Calculated, N 7.82; found, N 7.69

10 gm. of the salt were dissolved in 75 cc. of water and the solution was made acid to Congo red by the addition of HCl. The easily crystallized carbobenzoxy-d- β -amino-n-butyric acid was removed by filtration and washed with water. 5.8 gm. of material which melted at 110° were obtained. On analysis the compound gave the following values.

$C_{13}H_{15}NO_4$. Calculated, N 5.90; found, N 5.81

The carbobenzoxy group was removed by catalytic reduction and *d*- β -amino-n-butyric acid was obtained in good yield. The free acid had a specific rotation of $[\alpha]_D^{25} = +34^\circ$. This value is

¹ All melting points given in this paper are corrected.

in agreement with the value obtained by Fischer and Scheibler (3), $[\alpha]_D^{20} = +35^\circ$.

The combined ethyl acetate mother liquors from the crystallization of the *l*- α -phenylethylamine salt of carbobenzoxy-*d*- β -amino-*n*-butyric acid were concentrated to dryness and the residue was dissolved in 500 cc. of water. The solution was acidified and 33 gm. of partially active carbobenzoxy-*l*- β -amino-*n*-butyric acid were obtained. This partially active material was then treated with *d*- α -phenylethylamine salt, just as the racemic mixture had been treated before with *l*- α -phenylethylamine. The *d*- α -phenylethylamine salt of carbobenzoxy-*l*- β -amino-*n*-butyric acid was obtained. After two recrystallizations from ethyl acetate, it melted at 114° . The following analytical values were obtained.

$C_{20}H_{26}N_2O_4$. Calculated, N 7.82; found, N 7.76

Carbobenzoxy-*l*- β -amino-*n*-butyric acid was prepared from the salt in the same manner as the corresponding isomer. The compound melted at 110° , and gave the following analytical values.

$C_{13}H_{16}NO_4$. Calculated, N 5.90; found, N 6.05

l- α -Amino-*n*-butyric acid could be obtained from the carbobenzoxy derivative by catalytic reduction. The free amino acid had a rotation of $[\alpha]_D^{20} = -34.5^\circ$ for a 1 per cent aqueous solution. Fischer and Scheibler report a rotation of $[\alpha]_D^{20} = -35^\circ$ for this compound.

Preparation of Carbobenzoxy-d- β -Amino-n-Butyryl-l-Histidine and Carbobenzoxy-l- β -Amino-n-Butyryl-l-Histidine—Coupling of the carbobenzoxy derivatives of *d*- and *l*- β -amino-*n*-butyric acid chlorides with histidine methyl ester was carried out according to the directions given for the preparation of carbobenzoxyglycyl-*l*-histidine (2). The carbobenzoxy acid chlorides of *d*- and *l*- β -amino-*n*-butyric acid were stable for several hours at room temperature, in contrast to the corresponding derivative of glycine.

8.5 gm. of carbobenzoxy-*d*- β -amino-*n*-butyryl-*l*-histidine were obtained from 16 gm. of histidine methyl ester. The compound possessed a rotation of $[\alpha]_D^{25} = +28^\circ$ for a 1 per cent aqueous solution. The melting point was 204° . For analysis the com-

ound was recrystallized once from alcohol and dried at 100° *in vacuo*.

$C_{11}H_{21}N_4O_5$. Calculated, N 14.97; found, N 14.69

The corresponding diastereoisomer, carbobenzoxy-*l*- β -amino-*n*-butyryl-*l*-histidine, was prepared by the same method. The compound crystallized readily from water or alcohol. The melting point was 207° and a 1 per cent water solution had a specific rotation of $[\alpha]_D^{25} = +17^\circ$. After recrystallization, the material was dried *in vacuo* at 100° for analysis.

$C_{11}H_{21}N_4O_5$. Calculated, N 14.97; found, N 15.09

Preparation of d- β -Amino-n-Butyryl-l-Histidine—For the preparation of the free peptide, 4 gm. of carbobenzoxy-*d*- β -amino-*n*-butyryl-*l*-histidine were dissolved in 20 cc. of water, containing 2 equivalents of H_2SO_4 , and reduced with hydrogen in the presence of palladium black. The catalyst was removed by filtration and the SO_4^{--} was then precipitated with $Ba(OH)_2$ and filtered. The filtrate was concentrated to dryness *in vacuo* and the residue was dissolved in the minimum amount of water. 5 volumes of alcohol were added to precipitate the peptide, which crystallized readily. The amount of *d*- β -amino-*n*-butyryl-*l*-histidine obtained was 2.1 gm. The peptide melted at 260° and had a specific rotation of $[\alpha]_D^{25} = +21^\circ$ for a 1 per cent aqueous solution. It possessed the following analytical values.

$C_{10}H_{16}N_4O_5$. Calculated, C 49.99, H 6.71; found, C 49.82, H 6.58

Preparation of l- β -Amino-n-Butyryl-l-Histidine—*l*- β -Amino-*n*-butyryl-*l*-histidine was obtained from the carbobenzoxy peptide in the same manner as its diastereoisomer. The compound possessed a melting point of 260° and a 1 per cent solution in water had a specific rotation of $[\alpha]_D^{25} = +8.4^\circ$. The following analytical data were obtained.

$C_{10}H_{16}N_4O_5$. Calculated, C 49.99, H 6.71; found, C 50.05, H 6.70

Preparation of Optically Active Forms of Carbobenzoxy-dl- β -Aminoisobutyric Acid—50 gm. of α -bromopropionic acid were dissolved in 100 cc. of water and 1 equivalent of Na_2CO_3 was added. 16 gm. of NaCN dissolved in 200 cc. of water were added to this solution. The mixture was heated at 60° for 1

hour and was then reduced with hydrogen in the presence of Raney's nickel catalyst. After the reduction was complete, the catalyst was removed by filtration and the filtrate was treated with carbobenzoxy chloride. The carbobenzoxy-*dl*- β -aminoisobutyric acid melted at 76°. For analysis, the compound was recrystallized once from alcohol by the addition of water and was dried at room temperature *in vacuo*.

$C_{12}H_{14}NO_4$. Calculated, N 5.90; found, N 6.07

For the resolution of carbobenzoxy-*dl*- β -aminoisobutyric acid, the salt of *l*- α -phenylethylamine was utilized as in the previously described resolution of carbobenzoxy-*dl*- β -amino-*n*-butyric acid. The salt of carbobenzoxy-*d*- β -aminoisobutyric acid was the less soluble of the two salts formed and crystallized out of solution when the mixture was cooled. It melted at 91°. After three recrystallizations, the pure compound which was obtained melted at 98°. 27 gm. of the salt were obtained from 47.4 gm. of the carbobenzoxy-*dl*- β -aminoisobutyric acid. The compound gave the following analytical data.

$C_{20}H_{24}N_2O_4$. Calculated, N 7.82; found, N 8.16

Free carbobenzoxy-*d*- β -aminoisobutyric acid was obtained from the *l*- α -phenylethylamine salt in the same manner as described for the preparation of carbobenzoxy-*d*- β -amino-*n*-butyric acid. It melted at 88° and a 5 per cent solution in glacial acetic acid had a specific rotation of $[\alpha]_D^{25} = -6^\circ$. On analysis the following values were obtained.

$C_{12}H_{14}NO_4$. Calculated, N 5.90; found, N 6.00

Carbobenzoxy-*l*- β -aminoisobutyric acid was isolated by means of the *d*- α -phenylethylamine salt, as previously described for the isolation of carbobenzoxy-*l*- β -amino-*n*-butyric acid. The salt melted at 98°, and possessed the following percentage composition.

$C_{20}H_{24}N_2O_4$. Calculated, N 7.82; found, N 8.01

Carbobenzoxy-*l*- β -aminoisobutyric acid melted at 88° and a 5 per cent solution in acetic acid had a rotation of $[\alpha]_D^{25} = +6^\circ$. Analysis of the compound gave the following data.

$C_{12}H_{14}N_2O_4$. Calculated, N 5.90; found, N 5.94

Preparation of d- β -Aminoisobutyryl-l-Histidine—The conversion of carbobenzoxy-*d*- β -aminoisobutyric acid to the carbobenzoxy peptide by condensation with histidine methyl ester was carried out as previously described for the condensation of carbobenzoxyalanine with histidine methyl ester (1). Neither the carbobenzoxy acid chloride nor the carbobenzoxy peptide could be obtained in crystalline form. Reduction was, therefore, carried out directly on the oily carbobenzoxy peptide. *d*- β -Aminoisobutyryl-*l*-histidine was isolated from the reduction mixture as the copper salt. From 16 gm. of histidine methyl ester dihydrochloride, 2.1 gm. of the copper salt of the peptide were obtained. The salt melted at 230°. The air-dried material was rose-colored but, after the compound had been dried at 100° *in vacuo*, the color changed to a deep blue. The free peptide was obtained from the copper salt, as previously described for the preparation of *d*(-)-alanyl-*l*(-)-histidine (1). *d*- β -Aminoisobutyryl-*l*-histidine was somewhat soluble in aqueous alcohol and repeated concentrations of the mother liquor were necessary to obtain the crystalline peptide. It melted at 135° and had a specific rotation of $[\alpha]_D^{24} = +18^\circ$ for a 1 per cent aqueous solution. The air-dried material contained 2 molecules of water of crystallization. This compound and the blue-colored copper derivative possessed the following analytical values.

$C_{10}H_{18}N_4O_8 \cdot 2H_2O$.	Calculated.	C 43.48, H 7.30
	Found.	" 43.24, " 7.27
$C_{10}H_{16}N_4O_8 \cdot CuO$.	Calculated.	" 37.55, " 5.04
	Found.	" 37.77, " 4.81

Preparation of l- β -Aminoisobutyryl-l-Histidine—The oily acid chloride of carbobenzoxy-*l*- β -aminoisobutyric acid was condensed with histidine methyl ester by the method utilized for the preparation of carbobenzoxyalanyl-*l*-histidine (1). Carbenzoxy-*l*- β -aminoisobutyryl-*l*-histidine, like its diastereoisomer, could not be isolated in crystalline form. After reduction of the carbobenzoxy peptide, *l*- β -aminoisobutyryl-*l*-histidine was isolated as its copper salt, which melted at 205°. The free peptide melted at 240° and a 1 per cent water solution possessed a specific rotation

of $[\alpha]_D^{25} = +2^\circ$. *l*- β -Aminoisobutyryl-*l*-histidine and its copper salt gave the following analytical values.

$C_{10}H_{16}N_4O_4 \cdot CuO$.	Calculated.	C 37.55, H 5.04
	Found.	" 37.54, " 5.13
$C_{10}H_{16}N_4O_4$.	Calculated,	N 23.33; found, N 23.19

SUMMARY

d- β -Amino-*n*-butyryl-*l*-histidine and its diastereoisomer have been synthesized by condensation of the acid chlorides of the carbobenzoxy derivatives of *d*- and *l*- β -amino-*n*-butyric acid with histidine methyl ester. The free peptides were both isolated in crystalline form. Crystalline *d*- β -aminoisobutyryl-*l*-histidine and *l*- β -aminoisobutyryl-*l*-histidine were also prepared.

The peptides were tested for their effect on the blood pressure of cats under amytal anesthesia. Not one possessed any depressor activity in 20 times the effective dose of *l*-carnosine.

Conclusions from this and previous studies with regard to the relationship of structure to the depressor action of carnosine have been discussed.

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STUDIES ON THE NATURE OF THE IODINE IN BLOOD*

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Iodine metabolism is known to be significant in the maintenance of normal body function and is involved in certain pathological conditions. The rôle of the blood iodine in iodine metabolism can be understood only through a knowledge of the type or types of compounds present. In this study an attempt has been made to determine the nature of the blood iodine by means of comparing it with inorganic iodides, simple organic iodine compounds (thyroxine and diiodotyrosine), and iodine-containing protein. Comparisons have been made with regard to solubility, ultrafiltrability, and precipitation with protein precipitants.

Both human and animal bloods have been used in this study. The human blood samples and those from dogs were obtained by venipuncture, and extreme precautions were taken against the possibility of iodine contamination from the needle, syringe, anticoagulant, or the glassware used. The various blood samples were not obtained during the fasting state, but care was exercised to eliminate all persons who used iodized salt or who had eaten foods reported to be high in iodine content. In no case was a sample taken if iodine in any form had been used by the individual during the preceding 2 weeks. Animal bloods were obtained at the abattoir directly from the animal at the time of decapitation. The animals used had received a diet of grain, hay, and water and were known not to have received iodized salt for at least 10 days before the blood was taken.

A series of analyses of whole blood and plasma showed a dis-

* Material given in this paper is taken from a thesis presented to the Faculty of Cornell University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

tribution of the total iodine in proportion to the water content of the plasma and cells. Although it is recognized that a qualitative difference may exist between the plasma and cell iodine, there has been no evidence that such is the case. Whole blood and plasma have been used interchangeably according to the ease of manipulation. Iodine analyses were carried out according to the method of Trevorrow and Fashena (1, 2).

EXPERIMENTAL

Ethyl Alcohol Extraction of Blood with and without Addition of Iodine Compounds—Ethyl alcohol was used to extract the blood iodine, as shown in Table I. The blood was precipitated with 4 volumes of purified 95 per cent ethyl alcohol and the precipitate so obtained washed three times with 1 volume of alcohol. These four portions of alcohol were combined for analysis. The precipitate was then transferred quantitatively to a Soxhlet apparatus and extracted for 4 hours with fresh alcohol according to the method of Lunde, Closs, and Pederson (3). This alcohol was analyzed separately. The extraction was then continued for 24 hours and a third alcohol fraction so obtained. The blood protein residue was then analyzed for alcohol-insoluble iodine.

As can be seen in Table I, the iodine of blood is completely extracted by this procedure, 30 to 50 per cent being removed in the first fraction, 30 to 45 per cent in the 4 hour continuous extraction, and the remainder in the next 24 hours. The recoveries in these experiments ranged from 90 to 108 per cent and the protein residue in no case contained detectable amounts of iodine. Dodds and coworkers (4) have reported a long continued extraction of blood with hot alcohol and state that some of the blood iodine remains in the insoluble protein fraction. We believe these results to be in error as a result of an inadequate method of iodine analysis.

In similar extraction experiments in which potassium iodide, thyroxine, and finely minced thyroid gland were added to the blood, it was found that both the iodide and thyroxine were removed slowly by alcohol but that the thyroid iodine remained insoluble. It would appear, therefore, that the iodine of blood is not in protein combination similar to that found in the thyroid gland. In the last two experiments cited in Table I, blood con-

taining 2.8 micrograms of iodine and a similar sample of the same blood to which 26.0 micrograms of thyroid iodine had been added were extracted simultaneously under entirely comparable conditions. The iodine removed in the three alcoholic fractions was identical in amount in the two cases (within the limits of error of the method).

Acetone Extraction of Blood with and without Addition of Iodine Compounds—Acetone was next used for the extraction of the

TABLE I
Extraction of Blood Iodine with C_2H_5OH

Material used	I ₂ present	I ₂ in C_2H_5OH from precipitation and three washings	I ₂ in C_2H_5OH from Soxhlet extraction		I ₂ in residue after extraction	Recovery
			First 4 hrs.	Next 24 hrs.		
	micrograms	micrograms	micrograms	micrograms	micrograms	per cent
50 cc. dog blood	2.5	1.4	0.8	0.5	<0.1	108
50 " human blood	2.8	1.1	0.8	0.6	<0.1	90
50 " " + thyroxine	2.8 + 12.7	9.1	2.3	2.9	1.6	103
50 cc. human blood + KI	2.8 + 12.3	10.0	2.3	1.3	0.4	93
357.7 mg. thyroid	179.0		5.4	6.2	161.0	97
50 cc. beef blood + thyroid	2.8 + 26.0	0.8	1.3	0.6	*	
50 " " "	2.8	0.9	1.2	0.5	<0.1	93

* Not analyzed.

blood iodine, as shown in Table II. 1 volume of blood was added to 4 volumes of purified acetone and the mixture shaken vigorously in a mechanical shaking machine for from 1 to 12 hours as shown in Table II. The mixture was then centrifuged and the precipitate again shaken with 4 volumes of 80 per cent acetone. Four extractions were made in each case. The results are in accord with those of Eufinger and Schulte (5); namely, that all of the iodine is acetone-soluble. The time required for complete extraction was found to vary with the amount of iodine and the volume of blood used. 12 hour extraction periods served to extract completely 18 micrograms of iodine from 600 cc. of beef blood, whereas 3 hour extraction periods removed only 45 per

cent of the iodine from a similar 600 cc. sample. With smaller amounts of blood containing only 2 micrograms of iodine, 1 hour extraction periods were adequate.

Thyroxine and potassium iodide, when added to blood, were removed by acetone. In the 1 hour extraction periods used, only 72 and 86 per cent respectively of the iodine of these samples was dissolved by the acetone, but there can be no doubt that each of the added materials was being dissolved and would eventually have been completely removed from the precipitate.

The extracts obtained by this procedure were protein-free, as shown by the biuret test on a concentrated aqueous suspension of

TABLE II
Extraction of Blood Iodine with Acetone

Material used	Iodine present	Iodine in acetone		Iodine in residue		Time for each extraction*
	micrograms	micrograms	per cent	micrograms	per cent	
Beef blood	2.7	2.7	100	<0.2		1
“ “ + KI	1.3 + 3.0	3.7	86	0.6	14	1
“ “ + thyroxine	1.3 + 3.0	3.1	72	1.1	26	1
Human blood	2.4	2.4	100	<0.2		1
Beef blood	18.0	8.1	45	†		3
“ “	18.0	17.2	96	†		12

* Four extractions were performed in each case.

† Not analyzed.

the extracted material from which the acetone had been removed. This finding supports the view that the blood iodine is not in protein combination, but no differentiation can be made between simple organic iodine (thyroxine) and inorganic iodine.

Ultrafiltration of Blood with and without Addition of Iodine Compounds—Attempts were made to secure a protein-free preparation of the blood iodine by means of ultrafiltration. Theoretically, this procedure should give a simple aqueous solution of the blood salts and all simple organic compounds such as thyroxine and other amino acids, urea, sugar, etc. The membranes used were made from a 5 per cent solution of nitrocellulose (parlodion) in glacial acetic acid, deposited on the filter cone of a Giemsa filtration apparatus. This concentration of nitrocellulose is adequate

for the retention of all protein material of plasma. Leaks in the membrane surfaces were avoided by the use of a double thickness of collodion on each tube, the second layer being deposited after the first had been hardened in water. Each membrane was washed with several hundred cc. of redistilled water until the acetic acid had been removed and a 50 cc. sample of the filtrate showed no trace of iodine. After being washed and found iodine-free, the membranes were dried as completely as possible by suction, and used immediately.

Aqueous solutions of potassium iodide and thyroxine were ultra-filtered and the filtrate (after the first portions were discarded because of unavoidable dilution by water in the membrane) was found to contain the same concentration of iodine as that of the unfiltered material. Preliminary experiments with plasma and serum showed that no appreciable amount of the iodine present passed through the filter. The technique was then altered in such a way that a maximum amount of filtrate would be obtained from a given sample of plasma. 25 to 100 cc. of plasma were filtered until the non-filtrable residue had been reduced to one-fifth the original volume. Water was then added to the residue and the volume again reduced. The process was repeated a third time and the combined filtrates so obtained were analyzed as a whole. By this means 90 to 99 per cent of the filtrable iodine should be removed from the protein residue. The protein residues were also analyzed, as shown in Table III. The total iodine recovered ranged from 83 to 105 per cent, the low values presumably being due to a loss of protein residue which was thick, tenacious, and difficult to remove from the membrane.

As can be seen in Table III, the filtrates of blood plasma, serum, and laked cells showed no detectable iodine except in one instance in which 17 per cent of the total serum iodine appeared to be filtrable. Inorganic iodine was recovered quantitatively in the filtrate. Thyroxine and diiodotyrosine, although filtrable in simple aqueous solution, were retained in the unfiltrable residue after addition of an aqueous solution of the compounds to serum or plasma. Thyroxine added to a solution of egg albumin was also rendered non-filtrable, indicating that the property is not specific for blood but probably represents a simple non-specific adsorption of thyroxine by protein.

Ultrafiltration of certain protein solutions can therefore be used to differentiate between inorganic iodine and the iodine of thyroxine or diiodotyrosine. The results of this study of blood lead

TABLE III
Behavior of Blood Iodine, Potassium Iodide, Thyroxine, and Diiodotyrosine toward Ultrafiltration

Material used	Iodine present	Filtrate iodine	Non-filtrable iodine
	micrograms per 100 cc. H_2O	micrograms per 100 cc. H_2O	
Aqueous KI	20	19	
" "	20	21	
" thyroxine	90	94	
" "	74	74	
" "	64	62	
" "	48	48	
Sheep serum + KI	8.0+105.0	100	
Beef plasma + KI	7.8+133.0	140	
" " + KIO_3	8.0+ 50.0	44	
	micrograms	micrograms	micrograms
Beef plasma	3.0	<0.2	3.0
" "	3.4	<0.2	3.0
" "	4.1	<0.2	3.4
" "	6.5	<0.2	6.2
" "	1.7	<0.1	1.6
" serum	6.8	<0.2	*
" "	13.6	2.4 (17%)	12.1
" plasma + 1 volume H_2O	1.7	<0.1	1.6
" " + 1 " "	2.1	<0.1	*
" cells + 1 volume H_2O	4.0	<0.2	3.5
" plasma + 1 volume thyroxine solution	3.8+46.5	<0.2	48.5
" " + thyroxine	1.0+19.5	0.6	20.2
" " + "	2.3+17.0	<0.2	*
Egg albumin + "	2.9+ 9.6	<0.2	*
Sheep serum + diiodotyrosine	3.6+44.0	0.4	47.2
Beef plasma + "	1.4+12.0	<0.2	13.2

* Not analyzed.

one to conclude that the inorganic iodine constituents do not exceed 20 per cent of the total iodine and are usually less than 10 per cent or completely absent in so far as the present method permits their determination.

Since the completion of this work, Leipert (6) has published similar ultrafiltration experiments in which the filtrable iodine is said to constitute 60 to 90 per cent of the total. No analyses are given for the iodine content of the non-filtrable residue, this fraction being determined by difference only. The membranes used by Leipert were prepared from 6.5 to 7 per cent collodion in glacial acetic acid to which 2 per cent potassium carbonate had been added. The writer has prepared membranes of this type but has been unable to confirm Leipert's results. Leipert's method of iodine determination has been found by the writer (1) to be subject to variable positive errors which would partially account for the discrepancies between his results and those of the present study. Beef blood has been used in both studies. The results shown in Table III were obtained with blood from animals which had not received iodized salt. Four samples of blood from animals which had been fed iodized salt showed the filtrable iodine to range from 30 to 50 per cent of the total. It is possible that the blood used by Leipert may have been from animals on an iodine-rich diet which served to increase greatly the proportion of inorganic iodine present in the blood.

Use of Zinc Sulfate-Sodium Hydroxide and of Heat and Acetic Acid to Precipitate Blood with and without Addition of Iodine Compounds—Confirmatory evidence that the iodine of blood does not normally exist in inorganic form is shown in the following experiments. The proteins of whole blood were precipitated by two commonly used precipitating agents and the filtrates analyzed for iodine. Table IV shows the results obtained with the use of heat and acetic acid and Table V, those with zinc sulfate and sodium hydroxide. In neither case did the filtrate contain more than 10 per cent of the total iodine, and in both cases added iodide could be recovered in the filtrate and added thyroxine was retained to a large extent on the precipitate. Precipitation by heat and acetic acid was carried out according to the method of Benedict, Newton, and Behre (7), except that the treatment with colloidal iron was omitted; the zinc sulfate-sodium hydroxide precipitation, by the method of Somogyi (8). All reagents were iodine-free.

The results obtained by the use of alcohol and acetone extraction of blood have shown that the blood iodine probably does not exist in protein combination. Ultrafiltration and the experiments

with heat-acetic acid or zinc sulfate-sodium hydroxide precipitation of blood give evidence that not more than 10 per cent of the blood iodine is in inorganic form. Throughout the work, the

TABLE IV

Behavior of Blood Iodine Alone, and of Potassium Iodide and Thyroxine Added to Blood, toward Precipitation of Proteins with Heat and Acetic Acid

Material used	Iodine represented by filtrate	Iodine present in filtrate
	micrograms	micrograms
Beef blood	0.8	Trace
" "	1.4	<0.1
" "	1.9	Trace
" "	1.5	0.6 ?
" "	1.7	Trace
" " + thyroxine	1.5 + 4.2	0.7
" " + "	1.7 + 4.0	0.5
" " + KI	1.5 + 4.2	4.6
" " + "	1.7 + 4.0	3.3

TABLE V

Behavior of Blood Iodine Alone, and of Potassium Iodide and Thyroxine When Added to Blood, toward Precipitation of Blood Proteins with ZnSO₄ and NaOH

Material used	Iodine represented by filtrate	Iodine present in filtrate
	micrograms	micrograms
Human blood	2.2	<0.2
" "	3.7	<0.2
" "	3.4	Trace
" "	6.7	0.5
" " + thyroxine	0.3 + 1.8	<0.2
" " + "	0.2 + 2.0	Trace
" " + "	0.4 + 5.7	<0.2
" " + KI	0.2 + 2.6	2.4
" " + "	0.3 + 2.4	2.6
" " + "	0.4 + 6.3	4.0

blood iodine has behaved exactly as have added thyroxine and diiodotyrosine. The next problem is to show whether either or both of these iodine-containing amino acids may be present.

Analysis of Blood Extracts for Thyroxine by a Modified Leland and Foster Technique—Thyroxine can be determined chemically by the method of Leland and Foster (9) which is based upon the distribution of thyroxine between butyl alcohol and aqueous sodium hydroxide as contrasted with the solubility of other known iodine compounds in these solvents. This method was originally described for use with thyroid tissue, in which the ratio of thyroxine to organic material is much greater than that found in blood and the thyroxine is in protein combination. In the original

TABLE VI
Extraction of Blood Iodine with Butyl Alcohol

Material used	Iodine present Total iodine extracted		Distribution of total extracted iodine								
			Butyl alcohol			2 N NaOH					
			micro-grams	micro-grams	per cent	micro-grams	per cent of original	per cent of extracted	micro-grams	per cent of original	per cent of extracted
Human blood	18.0	20.0	111								
Beef blood	19.5	14.5	75								
“ “ + thyroxine	1.0	10.2	98								
	+9.4										
“ plasma + thyroxine	3.8	8.6	98	5.7				2.9			
	+5.0										
“ “	3.8	3.5	92	1.2	32	34	2.3	60	66		
“ “	71.0	31.8	45	19.2	27	60	12.6	18	40		
“ “	40.0	27.7	69	20.0	50	72	7.7	19	28		
“ “	35.0	27.4	79	18.0	51	65	9.6	27	35		
“ “	47.0	37.9	81	27.3	58	72	10.6	22	28		

method a long period of alkaline hydrolysis was employed to liberate the thyroxine from its combination in the protein molecule. During this treatment with alkali, approximately 20 per cent of added free thyroxine was found to be destroyed. Since the thyroxine in blood, if present, could not be in protein combination, it was deemed advisable to remove the protein by some means other than hydrolysis and thus avoid the destruction by alkali. This has been done by using butyl alcohol to extract the original blood. Table VI shows the results of these experiments. 1 volume of blood or plasma was added dropwise to 3 volumes of butyl alcohol and the mixture stirred gently, but constantly, for 15

minutes. Too vigorous mixing resulted in the formation of a thick emulsion which required as long as 24 hours for complete separation. This tendency toward emulsion formation was more marked with whole blood than with plasma, a finding which led to the exclusive use of plasma when large amounts were required. In cases where more than 100 cc. of plasma were extracted, the extraction was made on a series of 100 cc. portions, since this allowed for a more complete mixing of the plasma and butyl alcohol. After each extraction of a given sample, the mixture was centrifuged and the clear, yellow butyl alcohol removed from the aqueous phase and protein residue by decantation. 3 volumes of fresh butyl alcohol were added and the process repeated until all of the water (from the blood) had been removed and only a

TABLE VII

Distribution of Thyroxine, Diiodotyrosine, and Potassium Iodide between Equal Volumes of Butyl Alcohol and 2 N Sodium Hydroxide

Material used	Total iodine	Iodine in butyl alcohol	Iodine in NaOH	Volume of each phase
	micrograms	micrograms	micrograms	cc.
Thyroxine.	8.4	7.9	0.3	100
"	8.4	8.0	0.3	10
Diiodotyrosine . . .	8.0	0.4	7.5	10
KI.	8.0	0.6	7.2	10

finely granular residue remained. At this point the supernatant butyl alcohol was clear and colorless. From four to six extractions were made in each case.

Control experiments showed that all of the iodine of blood or plasma could be removed from small samples by this procedure and that added thyroxine could be recovered in the butyl alcohol. This again confirms the conclusion that the blood iodine is non-protein in nature. The butyl alcohol extract so obtained was concentrated under reduced pressure on a steam bath to a volume of 25 cc. for each 100 cc. of plasma used, and the concentrated butyl alcohol shaken with 1 volume of 2 N aqueous sodium hydroxide. Table VII shows the distribution of small amounts of pure thyroxine, diiodotyrosine, and potassium iodide between butyl alcohol and 2 N sodium hydroxide. The results are in agree-

ment with those reported by Leland and Foster (9) for larger amounts of material.

The mixtures obtained after shaking butyl alcohol extracts of blood with aqueous sodium hydroxide were allowed to separate completely and the two phases analyzed separately. As shown in Table VI, the butyl alcohol phase after this separation contained from 27 to 58 per cent of the total plasma iodine. Thyroxine when added to plasma was recovered in the butyl alcohol phase. In the last four experiments shown in Table VI, large amounts of plasma were used (500 to 1500 cc.), and the original removal of iodine from the protein was not complete. It is interesting to note that as the percentage of the total iodine removed increased in the different experiments, the increase was largely in the thyroxine-like fraction; the non-thyroxine iodine remained relatively constant at 20 to 30 per cent of the total plasma iodine. Too much emphasis should not be placed upon this finding, since in one experiment, in which 3.8 micrograms of iodine were completely removed from 50 cc. of plasma, 60 per cent was in a non-thyroxine form. It should be pointed out that this method, although added thyroxine can be recovered by it, may not be specific for this substance alone in the presence of iodine compounds other than diiodotyrosine and simple iodides. It is quite possible that the blood might contain some iodine compound other than thyroxine which has a high solubility in butyl alcohol as compared with its solubility in aqueous media.

The separation of the iodine into butyl alcohol- and water-soluble fractions is an actual separation of at least two different forms, as shown by the fact that the iodine of the butyl alcohol phase remains in the butyl alcohol with repeated extractions with 2 N sodium hydroxide and does not follow a percentage distribution of the order observed when the first separation was made. Were only a single compound present in the original extract, the aqueous sodium hydroxide would be expected to remove a constant percentage of the amount with each extraction.

Because of the non-specific nature of the above evidence for the presence of thyroxine, attempts were made to use biological tests on material obtained by the butyl alcohol and the acetone methods of extraction. This material, purified in various ways, has been used in the attempt to stimulate metamorphosis of tad-

poles. The results to date have been uniformly inconclusive because of the toxicity of the extracts. Further attempts to use methods of biological assay are planned for future work.

DISCUSSION

The results of this study are at variance with many of those reported in the literature, both in regard to factual material and the interpretation placed thereon. Part of this discrepancy may be due to positive errors in some of the methods of iodine analysis which have been used. As shown by us (1), Leipert's original method, in which arsenious oxide is used as the reducing agent, is subject to a variable positive error. We believe that all methods in which dry ashing is used to destroy organic material, alcohol extraction to remove the iodine from the ash, and final titration with sodium thiosulfate, are similarly subject to a positive error. This error results from the non-specificity of the Winkler titration in which any oxidizing agent capable of liberating iodine from potassium iodide or reacting with the thiosulfate will lead to erroneously high values. For example, in one of our experiments a portion of blood was extracted with ethyl alcohol until all of the original iodine had been removed and *could be accounted for in the extract*. The protein residue was analyzed by the Trevorrow and Fashena method and no iodine found. A second portion of the residue analyzed by the method of Turner (10) showed iodine to be present in an amount representing 4.0 micrograms per cent for the original blood. This type of error has therefore led to a general acceptance of the presence of "protein" iodine in the blood. Baumann and Metzger (11), in 1937, abandoned the dry ashing method previously published by them because of the variable positive errors involved.

That the method used in the present study is not subject to this error has been demonstrated in another way. 73 cc. of human blood were digested according to our analytical procedure and the iodine distilled. An aliquot of the distillate, representing 8.5 cc. of blood, was treated in the usual manner and iodine representing 5 micrograms per cent was found. The remainder of the distillate was treated with sodium bisulfite to reduce all of the iodine to iodide, evaporated to 2 cc., acidified, evaporated to 1 cc. (excess SO_2 boiled off), treated with palladium chloride, and the

color produced compared with that of standard iodide solutions. Iodine representing 5 micrograms per cent was found. Since the colorimetric procedure does not depend in any way upon oxidation or reduction reactions, the agreement between the results obtained by the colorimetric and titrimetric methods speaks well for the specificity of both procedures.

Besides the errors of analysis, we also believe that errors in interpretation have been made, especially with respect to the inorganic fraction of the blood iodine. Veil and Sturm (12), Lunde, Closs, and Pederson (3), and Eufinger and Schulte (5) have observed that a portion of the blood iodine can be extracted with solvents which remove inorganic iodides and have therefore concluded that this part of the blood iodine is inorganic in nature. These experiments have been inadequately controlled in that no consideration was given to the possible existence of simple organic iodine compounds. We have shown that the amino acids thyroxine and diiodotyrosine when added to blood can be extracted with these solvents.

By means of ultrafiltration and experiments with heat and acetic acid or zinc sulfate and sodium hydroxide precipitation, we have been able to differentiate between inorganic and simple organic iodine. The results of these studies lead to the conclusion that the blood samples studied cannot contain more than 20 per cent of their iodine in inorganic form.

SUMMARY

1. The iodine in the blood of animals on an iodine-poor diet is not in protein combination.
2. Not more than 20 per cent of this iodine is in inorganic form.
3. The greater part of the blood iodine possesses properties similar to those of thyroxine and diiodotyrosine, and a portion of this is not diiodotyrosine but is thyroxine-like in its solubility.

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THE ADSORPTION OF PROTEIN BY FILTER PAPER IN THE ESTIMATION OF ALBUMIN IN BLOOD SERUM

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The adsorption of albumin by filter paper during the determination of the albumin fraction of serum protein by the Howe method (1) has been discussed by Robinson, Price, and Hogden (2). They found that different filter papers adsorb correspondingly different amounts of albumin and so lower the concentration in the filtrate. They state, "The results prove that filter paper, in the presence of high salt concentration, does adsorb an appreciable amount of albumin and, therefore, the values for albumin are low, especially when small amounts of solution are filtered." The study deals with many kinds of filter paper and with repeated filtration through the same paper to observe the varying loss in albumin. The specific adsorption of particular papers did not receive comment but a survey of their data shows that Whatman No. 50 and Schleicher and Schüll No. 575 adsorbed less than the other papers; also that the loss due to adsorption could be eliminated by discarding the initial portion of filtrate.

In view of the above study, and of the routine use in this laboratory of the Howe method in albumin determination, 69 analyses were carried out in such a way that the loss of albumin could be appraised when one particular paper of good quality, Whatman No. 50, was used. The results corroborate the findings of Robinson and his associates in showing a loss in albumin due to adsorption. This loss, however, was exceedingly small and could be demonstrated only by applying statistical methods to the grouped data.

Method

The micro-Kjeldahl method of nitrogen determination as used in protein analyses in this laboratory has been adequately de-

scribed by Weech, Snelling, and Goettsch (3). Non-protein nitrogen, total protein, and albumin fractions are determined on each sample. The albumin fraction is estimated as that portion of the total protein left after the globulin has been precipitated by addition of a 22.2 per cent sodium sulfate solution and is the fraction which will be considered at this time.

2 cc. of dog serum were treated with 60 cc. of a 22.2 per cent solution of sodium sulfate at 37° as in the Howe micromethod and the mixture was well shaken. It was incubated at 37° overnight or for approximately 18 hours. The suggestions offered by Bruckman, D'Esopo, and Peters (4) for incubation and for maintenance of glassware at incubation temperature were followed. After incubation the mixture was again well shaken to distribute the precipitate evenly. The first 15 cc. portion was filtered through one 7.0 cm. sheet of Whatman No. 50 filter paper in a 38 mm., 60° angle glass funnel and collected. This is a smaller size of filter paper than that used by Robinson, Price, and Hogden, and does not hold the entire 15 cc. portion, but as it is the size used routinely in the laboratory, it was used in this study also. If the first 15 cc. portion was slightly cloudy, it was refiltered until clear; the remaining portion was then filtered through the same paper and collected separately. One analysis from the first 15 cc. portion and duplicate analyses from the remaining fraction were made by removal of 10 cc. aliquot portions, digestion, and distillation into $N/70$ HCl, with titration of the excess acid against $N/70$ NaOH. The same pipette was used to remove all aliquots. The transfers were accomplished as quickly as possible to prevent error due to evaporation and subsequent drying of the sodium sulfate mixture on the wall of the pipette. Digestion was accomplished by the addition of 4 cc. of a mixture containing 1 part of 5 per cent copper sulfate, 9 parts of distilled water, and 10 parts of nitrogen-free concentrated sulfuric acid to each flask.

The standard error of measurement (SE_M) for albumin analyses in this laboratory is ± 0.042 gm. per cent, as computed from 122 duplicate determinations both by the equation $SE_M = \sqrt{\Sigma d^2 / 2n}$ and $SE_M = (\Sigma d / 2n) \times 1.7725$, when d is the difference between duplicate values and n is their number (5). This error is slightly greater than the value of ± 0.03 gm. per cent given by Robinson and his associates. However, in calculating the standard error

these authors appear to have averaged a number of standard deviations (σ), each of which was computed from two, three, or four replicate analyses. This method of averaging σ rather than σ^2 tends to result in underestimation of the true value of the standard error. It is probable that analytical accuracy was about the same in the two laboratories.

Results

In order to evaluate the findings in the present study, the difference in the determined values for albumin concentration between

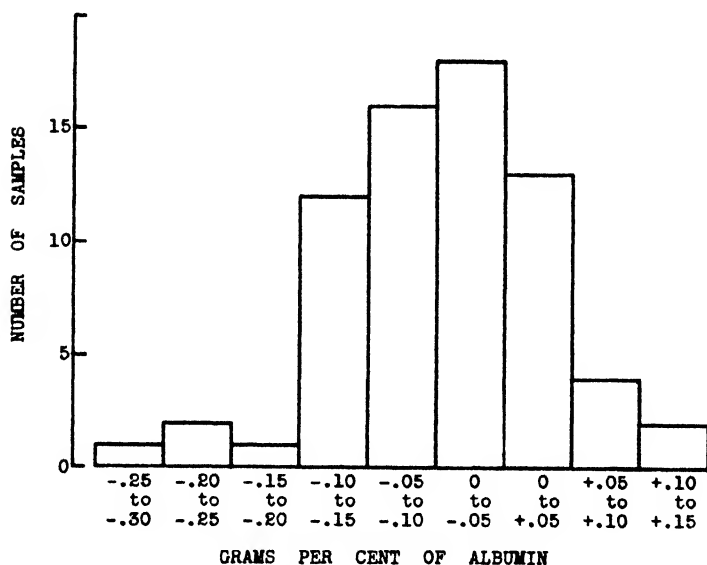


FIG. 1. Frequency polygon showing the distribution of the differences between determined values for albumin in initial and residual portions of filtrate.

the initial and residual portions of filtrate was calculated for each of the 69 samples. A negative sign was affixed to the difference when the initial filtrate yielded the lower result. In Fig. 1 the differences are arranged in the form of a frequency polygon and it is seen that the first portion of filtrate gave values which were sometimes lower and sometimes higher than the later portion. The extreme cases in the distribution reveal a total range among

the differences from -0.28 gm. per cent to $+0.13$ gm. per cent. The average difference with its standard deviation is -0.05 ± 0.0807 gm. per cent. The negative sign attached to this average figure is in accord with the belief that some albumin may be removed from the initial filtrate by adsorption. However, the magnitude of the average difference is surprisingly small and in view of the relatively wide scatter among individual differences it is reasonable to inquire whether or not there is any proof of adsorption by this filter paper. The proof is readily furnished by conventional statistical procedure. The ratio of the average difference to its probable error of ± 0.0065 gm. per cent is 7.7. This ratio is nearly twice as high as that required to prove that the average difference deviates from zero in the negative direction; that is, that the initial filtrate contains on the average less albumin than the later.

Since the data published by Robinson and his associates indicate considerable differences in the amount of adsorbed albumin when different types of filter paper are employed, it is not unreasonable to expect that some variability in adsorption will remain even when a single type of paper is used consistently. Perfect uniformity of a paper in this respect is scarcely possible and some causes of variation may reside in the serum rather than in the paper. Such circumstances may account in part for the range of values recorded in Fig. 1. Nevertheless it is reasonable to think that all papers must adsorb at least traces of albumin and the finding in numerous instances of a positive rather than a negative difference between initial and residual filtrate requires special explanation. It can be shown that this overlapping into the positive zone can be accounted for by the error of chemical analysis. The standard error of measurement for a single determination has been stated to be ± 0.042 gm. per cent. As the average of two analyses from the residual filtrate and a single analysis from the initial filtrate were involved in determining the difference between the two results, the standard error of measurement of the difference is ± 0.051 gm. per cent. This is calculated from the formula, $\sqrt{0.042^2 + \frac{1}{2}(0.042)^2}$, where 0.042^2 is the variance of a single determination and $\frac{1}{2}(0.042)^2$ is the variance of the average of two determinations. Since errors as great as 3 times the standard error of measurement are apt to occur in a

series of measurements, identical portions of the same filtrate, compared in a similar manner as in this study, may on analysis differ from each other by ± 0.153 gm. per cent on the basis of chemical error alone. In this series the greatest positive difference which was observed was $+0.13$ gm. per cent. As this value is not so high as the largest expected difference due to chemical error, it is evident that the positive values are included in the range of analytical deviation. There is no reason, therefore, to assume from this work that all filter papers do not adsorb at least traces of albumin. Moreover, many of the negative differences in Fig. 1 are much too large to be explained as errors of analysis, a circumstance which adds further support to the supposition that there is loss of albumin from the initial portion of filtrate.

Robinson, Price, and Hogden have suggested that the error due to adsorption can be eliminated by discarding the initial filtrate and analyzing only the residual portion. Under some circumstances samples of 0.5 cc. of serum must be analyzed. In such cases, when the total filtrate contains but 15.5 cc., elimination of the initial portion becomes impracticable. In a more recent paper Robinson and his associates (6) have suggested an alternate procedure which can be used in such cases and which depends upon substituting centrifugation for filtration. The findings in the present study provide another simple way of handling such samples. If the diameter and quality of the filter paper are the same as those employed in this study, the final analytical result can be corrected for loss due to adsorption by adding to it 0.05 gm. per cent of albumin. Since the adsorption losses with this type of paper are small, a constant correction applied to all samples will add very little to the total error which must continue to depend in large part upon other errors of analytical technique.

SUMMARY

69 samples of serum were analyzed for albumin by the Howe method in such a way that the loss of albumin due to adsorption by filter paper could be evaluated for one size and type of paper, namely Whatman No. 50 of 7 cm. diameter. The average loss of albumin was 0.05 gm. per cent. This figure although small possesses a high degree of statistical significance. It is shown that failure to obtain evidence of adsorption in individual analyses

can be explained on the basis of other errors of analytical technique. When small samples of 0.5 cc. of serum must be analyzed, an approximate correction for loss due to adsorption is provided by adding 0.05 gm. per cent to the final analytical result.

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PURIFICATION AND SOME PROPERTIES OF RENIN

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Vasopressor extracts of renal cortex were first prepared by Tigerstedt and Bergman (1) in 1898 and the active substance in them named renin. Further purification was carried out by Bingel and Strauss (2) and recently by Hessel and coworkers (3-5). Others (6-11) have also proposed methods which yield crude but pharmacologically active extracts.

In view of the renewed interest in this substance as the possible effector substance responsible for hypertension of renal ischemic origin (12-14) it seemed desirable to attempt purification of the relatively impure extracts which have been employed for studying the physiological properties of this pressor substance.

Method of Preparation of Renin

1 kilo of finely ground, fresh, pig kidney cortex is mixed with 2000 cc. of ice-cold acetone and kept at 0° for 2 hours. The mixture is shaken occasionally. It is then filtered by suction; the solids are washed with ice-cold acetone, returned to the original flask, and treated again with 2000 cc. of ice-cold acetone. After 2 hours at 0° the mixture is again filtered by suction. The insoluble portion is washed twice with ice-cold acetone, twice with ether, dried in air, placed in a vacuum desiccator overnight, and finally passed through a powder mill.

200 gm. of the powder are shaken for 15 minutes in a shaking machine with 700 cc. of ice-cold 2 per cent NaCl solution. The residue is removed by centrifugation and the supernatant fluid filtered through Schleicher and Schüll's filter paper No. 1117½. The residue is reextracted with 300 cc. of 2 per cent NaCl solution and treated as before. The residue is discarded.

To the combined filtrates, 7.5 cc. of glacial acetic acid are added, which precipitates a large amount of inert protein and brings the pH to 4.5. This precipitate is removed by centrifuging and the filtrate adjusted to a concentration of 1 M potassium phosphate by addition of 3 M potassium phosphate solution of pH 6.5.¹ It is then centrifuged and the supernatant fluid made up to a concentration of 2 M potassium phosphate. The precipitate (Fraction B) is collected on a Buchner funnel provided with a No. 42 Whatman filter paper, a small amount of diatomaceous earth (Hyflo super-cel) being added to insure a clear filtrate.

This precipitate (Fraction B) is dissolved in 100 cc. of 0.9 per cent NaCl solution and the precipitation with phosphate repeated as described. The precipitate (Fraction C) which is formed at 2 M concentration of phosphate is dissolved in 25 cc. of 0.9 per cent NaCl solution. This solution, which exhibits strong pressor properties, is deeply pigmented but is free from depressor substances. The nitrogen content is 3.7 mg. per cc.

Further purification is accomplished by adding to Fraction C an equal volume of 10 per cent NaCl solution and glacial acetic acid to 5 per cent concentration. Practically all of the pigment and 90 per cent of the protein nitrogen are removed, leaving the pressor fraction in the filtrate. The filtrate is dialyzed in cellophane sacs against distilled water overnight and adjusted to pH 6.5 with 0.1 N NaOH which precipitates further inactive proteins. The resulting solution (Fraction D) is water-clear and has a nitrogen content of 0.4 mg. per cc. From Fraction D, reineckates and picrates can be prepared which are strongly pressor. Ammonium sulfate concentration of 40 per cent of saturation at pH 3 to 4 and sodium chloride at full saturation at the same pH will also precipitate active pressor substance.

The procedures described above, except centrifuging, are carried out in the cold with ice-cold solvents. The whole process can be completed in 48 hours.

¹ This solution, which is made up of equimolecular amounts of monobasic and dibasic potassium phosphate, is prepared in 2 liter lots as follows: 817 gm. of KH_2PO_4 are weighed out in a beaker and 1 liter of water is added. Then 750 cc. of 4 N KOH are added and the solution warmed gently. When the phosphate is dissolved, the solution is transferred to a 2 liter volumetric flask, cooled to room temperature, and made up to volume.

Properties of Renin

Renin prepared by this method is stable for at least 2 months when kept cold. Heating at 50–55° for 15 minutes does not destroy its activity. Above 56° the activity is destroyed. Prolonged dialysis in cellophane membranes against distilled water causes precipitation of much of the pressor material, which is active when redissolved in dilute sodium bicarbonate solution. The accompanying pigment is also precipitated along with the

TABLE I
Color Reactions of Renin

Reaction		Result
Biuret	Peptide linkage	+
Ninhydrin	Free carboxyl and amino group	+
Millon	Tyrosine	+
Xanthoproteic	Benzene nucleus	+
Acree-Rosenheim	Tryptophane	—
Ehrlich's benzaldehyde		+
Adamkiewicz		+
Ehrlich's diazo	Histidine, tyrosine	+
Sakaguchi	Arginine (guanidine group)	++
Reduced sulfur	Cystine, cysteine	+
Molisch	Carbohydrate	+
Bial's orcinol	Pentose	++
Dische's indole	"	++
Naphthoresorcinol	Glucuronates	
Phloroglucinol	"	
Diphenylamine	Levulose, nitrates, nitrites	
Folin's phosphotungstic	Adrenine	
Bromocyanogen	Pyridine	

renin on dialysis. Renin does not pass through a 3 per cent collodion ultrafilter membrane, whereas the pigment does. It is precipitated readily from solution by Reinecke salt and by picric acid, and these precipitates are highly pressor when injected into animals.

Color reactions of Fraction D are shown in Table I. It is interesting that the Sakaguchi test for arginine and guanidine groups and the Bial and Dische tests for pentose are strong, while the reaction for adrenine is negative. The hydrolysate of Frac-

tion D forms precipitates with ammoniacal silver nitrate, flavianic acid, picric acid, and Reinecke salt.

Fraction C has been used for pharmacological studies. When injected intravenously into an unanesthetized dog, 0.025 cc. per kilo will produce a rise in blood pressure of 30 mm. or more, as measured by a mercury manometer connected to an intraarterial cannula. It is also highly active when injected into cats or rabbits anesthetized with pentobarbital. 6 or 7 cc. perfused into a dog over a period of an hour will cause a large increase in blood

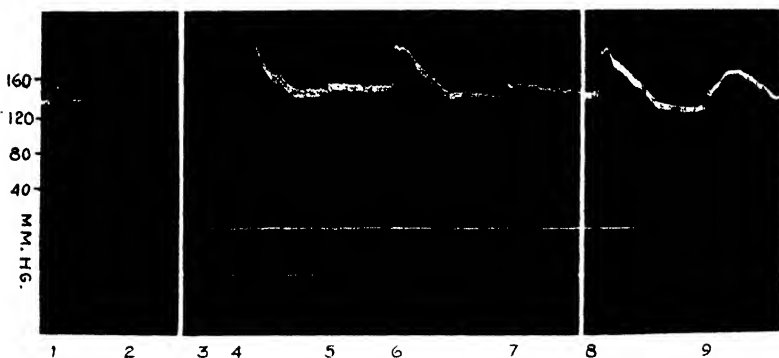


FIG. 1 Arterial blood pressure of a cat weighing 3.1 kilos, anesthetized with pentobarbital: 1, represents 1 cc. of adrenne (1:100,000); 2, renin 0.2 cc. (1:1 dilution); 3, cocaine 6 mg.; 4, adrenne 1 cc.; 5, renin 0.2 cc.; 6, adrenne 1 cc.; 7, renin 0.4 cc.; 8, adrenne 1 cc.; 9, renin 0.8 cc. Pressor response to renin before cocaine 10 mm. of Hg and after, 8 mm. of Hg for the same dose, 16 mm. of Hg for twice the dose, and 40 mm. of Hg for 4 times the dose.

pressure without lowering the skin temperature (9). Fraction D produces a rise of 30 mm. in arterial pressure of dogs in quantities representing 0.04 mg. of nitrogen per kilo of body weight. The precipitate formed by saturating Fraction D with sodium chloride at pH 4 causes a similar rise in blood pressure when injected in quantities representing 0.027 mg. of nitrogen per kilo of body weight. In cats 0.009 mg. of renin nitrogen produced a rise of blood pressure of 32 mm. of Hg. This is the most active renin which we have prepared.

To ascertain whether the pressor action of renin prepared in

this manner was potentiated by cocaine, as is adrenine, cats were anesthetized with pentobarbital and the arterial pressure recorded from the carotid artery after section of a vagus nerve. Control injections of adrenine (1 cc. of 1:100,000 solution) and renin were given, followed by injection of cocaine (2 mg. per kilo of body weight). After demonstration of marked potentiation of the pressor action of adrenine, renin was again injected. No potentiation was observed (Fig. 1).

Doses of ergotamine sufficient to depress markedly the pressor action of adrenine did not abolish the pressor effect of renin. These results add no support to the view that the pressor action of renin is due to adrenine or adrenine-like substances.

It is important to note that the response of cats to 2 and 4 times the original dose is proportional to the dosage. For this reason cats make desirable animals for assay purposes. Assay of renin by this method will be discussed in detail in a forthcoming communication.

DISCUSSION

Renin is doubtless a protein, as Tigerstedt and Bergman (1) recognized. The fact that the purer the preparation the less active it is when perfused through a dog's tail with Ringer's solution and that it is activated by addition of serum or colloids from serum led Kohlstaedt, Helmer, and Page (15) to suggest that renin is an enzyme-like substance. It is a protein, inactivated by heat and, when relatively pure, activated by blood colloids.

Discussion has arisen as to whether the pressor quality of renin is due to the molecule as a whole or to some prosthetic group within it. Williams (16) found that the rise in blood pressure produced by partially purified extracts in white rats is enhanced by cocaine and antagonized by ergotamine. Since adrenine is similarly affected, he suggested that the prolonged rise of arterial pressure is due to gradual liberation of an adrenine-like substance. Hessel (5) and Friedman, Abramson, and Marx (17) found no especial potentiation of renin by cocaine in dogs. Nor did Hessel find that ergotamine prevented its action. We have repeated these observations on dogs and cats with our purified renin and find no potentiation with cocaine nor

reversal to depressor action after preliminary treatment with ergotoxin.³

Against the view-point that the action of renin is due to slow liberation of adrenine is the observation of Landis, Montgomery, and Sparkman (9) that renin is the only substance among the usual pressor agents, such as adrenine, which produces a rise in blood pressure without lowering the skin temperature.

Purified renin does not give the Folin color reaction for adrenine. Neither the pharmacological nor chemical evidence supports the view that the pressor action of renin is due to gradual liberation of adrenine. Although the blood pressure rise is somewhat similar to that produced by tyramine, it has been shown by Williams, Harrison, and Mason (11) that the pressor response of renin is not abolished by cocaine, as is that of tyramine.

An alternative explanation might be offered; namely, that the prolonged action is due to gradual activation of renin by the activator in blood (15).

SUMMARY

1. A method for purification of renin, a pressor substance from kidney cortex, has been described. The purest preparations elevate arterial blood pressure 30 mm. of Hg in dogs when injected in amounts representing 0.027 mg. of nitrogen per kilo of body weight. In cats it was 3 times as active; namely, 0.009 mg. of nitrogen-containing extract produced the same rise.

2. Color tests for guanidine groups and for pentose are especially strong in renin solutions; those for adrenine are negative. Crystalline precipitates are formed on addition of flavianic acid or picric acid to hydrolysates of renin.

3. The pressor action of renin in cats and dogs is not abolished by ergotamine nor potentiated by cocaine as is adrenine.

³ Dr. John Williams, Jr., in a letter kindly suggests that the differences in our results may be due to the fact that (1) different animals were used, (2) different methods for the preparation of renin were employed. His results may be due to the adrenine-like substance which he finds in the kidneys and not to renin. Since he finds rats proportionately more sensitive to adrenine than cats and dogs, the adrenine-like substance may have overshadowed the renin action. Apparently none of this adrenine-like substance is present in our purified renin.

Cats anesthetized with pentobarbital are desirable preparations for assay purposes.

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THE FATE OF TRICHLOROETHYLENE IN THE ORGANISM

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During the course of an investigation of the toxicity of trichloroethylene and carbon tetrachloride (1) it was observed that trichloroethylene underwent some change in the body.

Brüning and Schnetka (2) employed the Fujiwara (3) reaction to determine the distribution of trichloroethylene in the tissues of animals that had been exposed to the vapor. They also reported the presence of trichloroethylene in the urine of human subjects who had undergone trichloroethylene exposure.

The Fujiwara reaction occurs on addition of pyridine and sodium hydroxide to dilute solutions of many halogenated hydrocarbons or their derivatives. In this laboratory the reaction was adapted for the determination of trichloroethylene vapor in air (4), the standard solutions being prepared in 20 per cent alcoholic solution. When the steam distillate of urine obtained from human subjects who had been exposed to trichloroethylene vapor was tested in 20 per cent alcohol, it was found that the color that developed in the urine distillate after prolonged heating was a distinct lemon-yellow, whereas solutions of trichloroethylene turned definitely orange. That this change in color was not produced by a reaction between urine and trichloroethylene or by some interfering substance was shown by adding trichloroethylene to normal urine and keeping it in sealed tubes for periods as long as a week. The steam distillates from these samples gave a color, when tested by the Fujiwara reaction, that was identical with that given by the standard trichloroethylene solutions.

Further evidence that the halogen-containing material present

in the urine following trichloroethylene exposure was not trichloroethylene was supplied by the following experiments. Trichloroethylene added to normal urine could be completely removed by passing air through the solution and the trichloroethylene could be absorbed from the air by absolute alcohol. Urine from exposed human subjects or animals showed no detectable decrease in halogen-containing material following the passage of air, and tests on the alcohol in the absorption tube were negative. Trichloroethylene added to normal urine could be completely removed by shaking with a small volume of ethyl ether when the urine was either acid or alkaline. On the other hand, the halogen-containing material present in urine obtained from exposed human beings or animals was practically insoluble in ether when the solution was alkaline but would dissolve if the urine had first been acidified. These experiments showed that not only was trichloroethylene changed by the organism, but also that no appreciable quantity of unchanged trichloroethylene occurred in the urine.

Since it was not feasible to obtain sufficient urine from exposed human subjects to attempt isolation of the material giving a positive Fujiwara reaction, dogs were employed. The active material that was present in dog urine following exposure of the animals to trichloroethylene vapor was indistinguishable from that present in the urine from exposed human beings in so far as the tests that have been outlined could show. Three dogs weighing about 15 kilos each were exposed to trichloroethylene vapor in a wooden box. The trichloroethylene was vaporized by passing compressed air through a flask containing the liquid. The dogs were completely narcotized (this usually required from 20 to 30 minutes) and were then removed from the box and allowed to recover. The animals were exposed once or twice a day, Sunday excluded. The urine from all animals was tested prior to the first exposure and showed the absence of any material giving a positive Fujiwara test. After a few exposures, sufficiently high concentrations of the material were present in the urine to enable Fujiwara tests to be performed on urine diluted 1:100 with water. At this dilution the color of the urine did not interfere appreciably with the tests. It was observed that the colors given in the Fujiwara test both by dilute urine and the steam distillate of urine were identical and furthermore were indistinguishable from the colors given by

solutions of chloroform. For this reason, aqueous solutions of chloroform containing 1, 2, 3, 4, and 5×10^{-6} part of chloroform by volume were used as standards to estimate quantitatively the efficiency of various means of concentrating the halogen-containing material present in the dog urine.

With Gettler and Siegel's method (5), about 15 mg. of chloroform were isolated from the steam distillate from 750 cc. of dog urine. The chloroform was purified by distillation *in vacuo* from concentrated sulfuric acid. It was identified by Weber (6) and Fujiwara color tests and by the following physical constants.

	B.p.	Mol. wt.	Refractive index	Density (15°/4°)
	°C.			
Material from steam distillate.....	59.5-61	121.1	1.4460	1.499
Chloroform.....	61.2	119.4	1.4464	1.498

The boiling point was determined by Emich's (7) method. The molecular weight was obtained by the method of Niederl and Saschek (8). An Abbe Zeiss refractometer was used to determine the refractive index and the density determination was made by a microdensity method developed in this laboratory (9) for determining deuterium oxide concentrations. The pycnometer used in the density determinations had a capacity of approximately 11 c.mm. It was weighed on a Kuhlmann balance.

It was found next that chloroform did not occur in the urine as such but resulted from the decomposition of trichloroacetic acid. This material was isolated from the urine by repeated extractions with ether from acid solution and from ethereal solution with alkali. A small amount of charcoal was used to decolorize the last alkaline extract. Purification was effected by distillation under reduced pressure. About 0.5 gm. of crystals was obtained from 13 liters of urine. The melting range was 54-57°; the molecular weight determined by neutralizing with 0.01 N NaOH was 162. The piperazonium salt was prepared by the method described by Pollard, Adelson, and Bain (10). This melted sharply at 121.5°. No depression occurred when the sample was mixed with an equal quantity of the piperazonium salt prepared from a known sample of trichloroacetic acid. A small sample of the sodium salt of the

material was precipitated from alcohol by the addition of petroleum ether. The composition of the two salts is shown in Table I.

The intensity and color of the Fujiwara tests given by the two salts prepared from the isolated material were identical with those given by equal concentrations of similar salts prepared from trichloroacetic acid.

TABLE I*

Per Cent Composition of Piperazonium and Sodium Salts from Dog Urine

	Carbon	Hydrogen	Nitrogen	Chlorine
Piperazonium salt of material from dog urine.....	23.19	2.96	6.88	51.31
Calculated for piperazonium dichloroacetate, $C_3H_{12}Cl_6N_2O_4$	23.26	2.91	6.79	51.53
			Sodium	
Sodium salt of material from dog urine.....	13.21	0.02	12.46	56.24
Calculated for sodium trichloroacetate, $C_2Cl_3NaO_2$	12.95	0.00	12.40	57.38

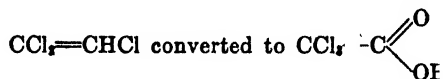
* We are indebted to Dr. G. F. Marrian and Miss D. Skill of the Department of Biochemistry for these analyses.

DISCUSSION

It has been shown that trichloroacetic acid can be isolated from the urine of dogs that have been exposed to the vapor of trichloroethylene. Steam distillation of this urine yields chloroform. It cannot, of course, definitely be said that trichloroacetic acid was not conjugated in some way or that it is not a decomposition product of some other material which in the process of isolation was broken up. In this connection the only other metabolic products resulting from the administration of chlorinated narcotic substances that have been positively identified are urochloralic acid and urobutylchloralic acid which are excreted following the administration of chloral and butyl chloral. In isolating urochloralic acid von Mering and Musculus (11) used methods somewhat similar to those employed in the present work. Urochloralic acid undergoes decomposition fairly easily on being boiled with

acid, yielding trichloroethanol and glucuronic acid. Therefore, if trichloroacetic acid results from decomposition of some other substance, that material must be extremely unstable.

The conversion of trichloroethylene to trichloroacetic acid cannot be easily explained by any at present known chemical reaction. As will be seen from an examination of the formulæ of the two materials, such a conversion entails the transfer of a chlorine atom from one carbon atom to the adjoining one.



Such a conversion might be brought about, however, by the addition of HCl to trichloroethylene with the formation of unsymmetrical tetrachloroethane; oxidation of this substance might yield trichloroacetic acid. Another possible, although seemingly improbable, explanation is that trichloroethylene acts as a chlorinating agent and produces trichloroacetic acid in this manner.

Although trichloroacetic acid was not actually isolated from the urine of human subjects exposed to trichloroethylene, owing to the impossibility of obtaining a sufficient supply of urine, it is strongly indicated that the same conversion occurs in human beings as well as dogs. A discussion of the significance of the conversion of trichloroethylene to trichloroacetic acid, from the standpoint of a detoxification mechanism, along with data regarding the fate of some other chlorinated hydrocarbons will be contained in another report.

SUMMARY

Trichloroacetic acid has been isolated from the urine of dogs that have been exposed to the vapor of trichloroethylene. The methods of isolation and identification of the material have been described. Experiments with urine from human subjects exposed to trichloroethylene vapor indicate that a similar conversion probably occurs in human beings as well as in dogs.

Thanks are expressed to Dr. C. H. Best for his interest in this investigation.

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THE ACTIVITY OF THE PHOSPHORYLATING ENZYME IN MUSCLE EXTRACT*

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It has been shown that various mammalian tissues (muscle, heart, brain, liver) as well as yeast contain an enzyme which can be extracted with water and which forms glucose-1-phosphoric acid (1-ester) from glycogen and inorganic phosphate (1). This enzyme, which will be referred to as phosphorylase, initiates the degradation of glycogen. The further fate of 1-ester depends on the nature of the enzyme systems which are present. In muscle 1-ester is converted to lactic acid, while in liver it is converted to glucose and inorganic phosphate (2). There is also the possibility that the phosphorylase initiates glycogen oxidation.

It was found (3) that the activity of the phosphorylase depends on the presence of minute amounts of muscle adenylic acid or of adenosinediphosphate, which act as coenzymes in an as yet unexplained manner. The present report deals with a more detailed study of the activity of this enzyme and the effects on it of specific ions (Mg, Mn) and of other substances.

EXPERIMENTAL

The experiments were carried out with dialyzed¹ and in some cases with electrodialyzed extracts of rabbit muscle, prepared

* This work was aided by a research grant from the Rockefeller Foundation.

¹ The effectiveness of the dialysis is shown by the following figures. The inorganic P content of the extracts in Experiments 1 to 6 in Table I was 1.1, 1.5, 4.0, 4.5, 3.5, and 4.1 mg. per cent, respectively; the acid-soluble organic P varied from 0.6 to 1.2 mg. per cent. In this and in previous work the collodion membranes were placed in water immediately after

as previously described (5). Besides phosphorylase, these extracts contained phosphoglucomutase (6) and isomerase (7) which by successive action convert the 1-ester to the equilibrium mixture of glucose- and fructose-6-phosphate (6-ester). The degradation of glycogen in these muscle extracts did not progress beyond the formation of 6-ester. No inorganic P was split off from 6-ester during 3 hours of incubation at 37°.

Phosphorylase activity was measured by the disappearance of inorganic P or by the isolation of the reaction products (1- and 6-esters) as the barium salts and their determination by a method previously outlined (1). As shown in a previous paper (3), good agreement is obtained between these two procedures. Glycogen was determined before and after incubation by heating aliquots of the reaction mixture for 30 minutes in 30 per cent NaOH and precipitating with 1.5 volumes of alcohol heated to the boiling point (Good *et al.* (8)). The glycogen was dissolved in water and reprecipitated with 2 volumes of alcohol before it was hydrolyzed in N HCl. After neutralization of the hydrolysate with NaOH, glucose was determined by the copper reduction method of Shaffer and Somogyi (1 gm. of KI reagent). Further experimental details are given in the Table I and Figs. 1 to 6.

Comparison of Glycogen Disappearance and Hexosemonophosphate Formation—Table I shows that after 10 to 30 minutes of incubation from 93 to 97 per cent of the glycogen which disappeared was accounted for by the formation of hexosemonophosphate which consisted mainly of 1-ester when no Mg^{++} was added to the reaction mixture. After 60 to 90 minutes of incubation the percentage of glycogen accounted for was somewhat lower and varied from 82 to 98 per cent. It seemed possible that a small amount of glycogen disappeared through diastatic action² and thus escaped phosphorylation.

their preparation; they were not hardened by drying or by treatment with dilute alcohol. The extracts were dialyzed for 18 to 22 hours against running tap water of about 10°. That an extended period of dialysis may be quite ineffective when less permeable membranes are used is shown by figures published by Augustin (4) for beef muscle extracts which contained 40 mg. per cent of inorganic P after 23 hours of dialysis in cellophane tubes.

² The first products of diastatic activity, the dextrans, can probably be phosphorylated by muscle extract, while the chief end-products of diastatic

In order to test the extracts for diastatic activity, inorganic P was omitted from the reaction mixture, or no adenylic acid was added as in Experiment 6, or the phosphorylase was inhibited by addition of phlorhizin. Under these conditions a small amount of glycogen still disappeared. Since in Experiments 1 to 6 the dialyzed extracts were not free of inorganic phosphate (see foot-note 1) and since some ester formation was still detectable, the disappearance of glycogen without addition of phosphate could have been due only in part to diastatic activity. In the extensively electrodialed extract (Experiment 7) there was practically no disappearance of glycogen during 3 hours of incubation when no phosphate was added. This extract formed large amounts of hexosemonophosphate during 10 minutes of incubation with phosphate buffer, so that it may be concluded that the phosphorylase disrupts the glycogen molecule without the aid of diastase. In discussing phosphorylase activity, Parnas (10) has expressed a similar view.

The rather close correspondence between glycogen disappearance and hexosemonophosphate formation leads to the conception that once a glycogen molecule is acted upon by the phosphorylase, it is completely broken down into uniform fragments consisting of 1-ester. This makes clear the dissimilarity of action of phosphorylase and diastase, since the latter enzyme causes a stepwise breakdown of the glycogen molecule.

Influence of Substrate and of Enzyme Concentration—In the reaction catalyzed by the phosphorylase two substrates, glycogen and inorganic P, are present, the concentration of each of which influences the rate of enzyme activity. Electrodialyzed muscle

activity, maltose and glucose, are not phosphorylated. Two dextrans designated as Dextrans D and F in Table II of a paper by Caldwell and Hixon (9) and for which average chain lengths of 74 and 26 glucose units respectively are given were kindly supplied to us for examination. Dextrin D, according to our analysis, contained 39.5 per cent of alkali-resistant material, when heated for 30 minutes in 20 per cent KOH, followed by precipitation from 50 per cent alcohol, Dextrin F only 3.9 per cent. Dextrin D was phosphorylated more rapidly in muscle extract than Dextrin F, but both were phosphorylated more slowly than glycogen. With both dextrans more hexosemonophosphate was formed than corresponded to the alkali-stable part.

TABLE I

Glycogen Disappearance and Hexosemonophosphate Formation

The reaction mixture consisted of 2 parts of dialyzed rabbit muscle extract and 1 part of additions; it contained $m/9$ phosphate buffer of pH 7.2, 1 per cent glycogen, 1 mm adenylic acid, and 10 mm $MgCl_2$, except as noted. The mixture was incubated at 25°, except as noted.

The values are given in mg. per 10 cc. of mixture.

Experiment No.	Period of incubation	Hexosemonophosphate formed (as hexose)			Glycogen disappearance (as hexose)	Glycogen accounted for	Remarks
		1-Ester	6-Ester	Total			
	min.					per cent	
1	30	5.0	1.1	6.1	6.3	97	No Mg added
	60	6.8	1.6	8.4	10.3	82	" " "
2	30	11.5	1.0	12.5	13.4	93	" " "
	60	11.1	3.4	13.5	16.4	82	" " "
3	60	0.3	9.9	10.2	11.7	87	
	60	0.5	1.0	1.5	2.8		10 mm phlorhizin
	60				3.2		No phosphate added
4	60	0.9	16.5	17.4	20.6	84	
	60	1.4	17.3	18.7	20.6	91	0.02 M maltose
	60				2.6		10 mm phlorhizin
5	60				3.3		No phosphate added
	60	11.7	7.2	18.9	19.2	98	" Mg added
	90	0.7	20.0	20.7	24.5	84	
6	60	0.6	2.5	3.1	3.7		10 mm phlorhizin
	60				4.1		No phosphate added
	10*			37.7†	40.7	93	
7†	20*			49.0†	52.8	93	
	10*			2.8†	4.7		No adenylic acid added
	20*			2.8†	7.8		" " " "
7†	10*			34.2†			
	20*			54.5†			
	60*				0.1		No phosphate added
	180*				0.7		" " "

* Incubated at 37°.

† Calculated from the disappearance of inorganic P.

‡ Electrolyzed extract.

extracts were used which did not contain the adenylic acid deaminase³ described by Schmidt (11), a factor which is of import-

³ The preparation of muscle extracts free of deaminase by aging or by charcoal treatment has been described in a previous paper (3). During aging the deaminase is found in an active state in the protein precipitate which settles out and it is necessary therefore to centrifuge or filter the extracts before use.

ance in an enzyme reaction in which adenylic acid acts as co-enzyme, particularly in a study of the kinetics of the reaction.

The effect of different phosphate concentrations, with all other conditions kept constant, is shown in Fig. 1. The lowest phosphate concentration in Fig. 1 was about twice that found in resting muscle. With $M/9$ phosphate the rate of activity was initially greater than with $M/18$ phosphate, but fell off more rapidly, so that after 1 hour of incubation the same amount

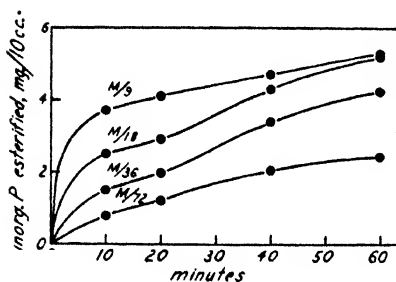


FIG. 1

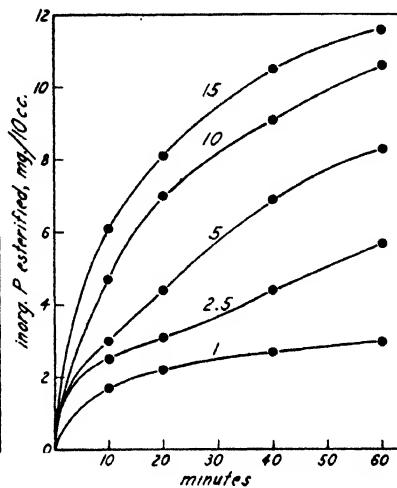


FIG. 2

FIG. 1. Effect of phosphate concentration. An electrodialyzed and 9 times diluted rabbit muscle extract was incubated at 37° with 1 per cent glycogen, 10 mM $MgSO_4$, 1 mM adenylic acid, and varying concentrations of phosphate buffer of pH 7.2, as marked on the curves.

FIG. 2. Effect of enzyme concentration. The same extract that was used in Fig. 1 was incubated at 37° with 1 per cent glycogen, $M/18$ phosphate, 10 mM $MgSO_4$, and 1 mM adenylic acid. The enzyme concentrations, expressed in arbitrary units, were 1, 2.5, 5, 10, and 15 respectively, as marked on the curves.

of phosphate was esterified with the two concentrations. After 2 and 3 hours of incubation (not included in Fig. 1) the esterification was less with $M/9$ and $M/4.5$ than with $M/18$ phosphate. The glycogen concentration was not a limiting factor, since practically the same curve was obtained with $M/18$ phosphate whether 1 or 2 per cent glycogen was used. It appears therefore

that $m/18$ phosphate and 1 per cent glycogen (*i.e.* equivalent amounts) were optimal for this particular enzyme concentration.

With these substrate concentrations ($m/18$ phosphate and 1 per cent glycogen) were tested one lower and three higher enzyme concentrations than the one used in Fig. 1, the total range of enzyme dilution being 15-fold. As shown in Fig. 2, there exists a fairly good proportionality between enzyme concentration and activity over this range; that is, the time to esterify a given amount of inorganic P is inversely proportional to the enzyme concentration. This relationship is shown in the following values for K ($K = TE$, where T is time in minutes and E enzyme concentration, the latter being expressed in arbitrary units of 1, 2.5, 5, 10, 15, respectively).

K for different amounts of P esterified			
	0.3 mg. P	0.6 mg. P	0.8 mg. P
1	60		
2.5	45	147	
5	50	151	285
10	52	160	275
15	52	158	275

This proportionality holds even though there is a rapid falling off in the rate of enzyme activity with time, which makes it difficult to measure initial rates. The reason for the falling off in rate has not been fully investigated. It was ascertained that an inactivation does not occur when muscle extracts are incubated for 1 hour at 37° with glycogen alone or phosphate alone before the other additions are made. There are several factors which influence the shape of the time curves. In extracts containing deaminase the rate of enzyme activity may fall off even more rapidly than in Fig. 2, owing to a diminishing and eventually suboptimal adenylic acid concentration. The same is true if no Mg^{++} is added, so that the inhibitory reaction product, the 1-ester, accumulates. The age of the extract has a marked effect on the shape of the time curves. These and other factors make it difficult to establish a unit of enzyme activity which will serve for different muscle extracts or for the same extract at different

times after its preparation. For a further study of its kinetics a purification of the enzyme seems necessary.

Effect of pH and Temperature—A pH-activity curve is shown in Fig. 3. The pH of the reaction mixture was determined by means of a glass electrode. The plateau between pH 7 and 8 was verified in an experiment with a different enzyme concentration, marked by crosses in Fig. 3. At pH 5.8 and 9 the enzyme activity was about one-half of that at the pH optimum. The influence of pH on the activity of the enzyme has recently been studied by Bauer *et al.* (12).

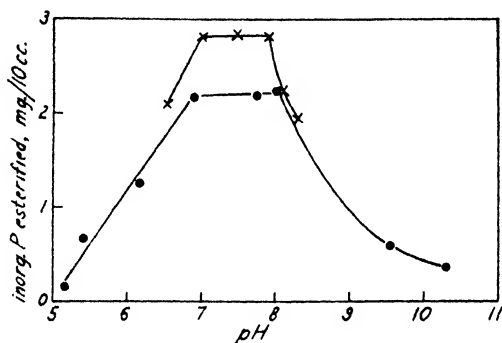


FIG. 3. pH-activity curve of phosphorylase. A rabbit muscle extract dialyzed for 18 hours and diluted 1.5 times was incubated with 1 per cent glycogen, $M/22$ phosphate buffer, and 0.1 mM adenylic acid for 30 minutes at 27°. Part of the curve, marked by crosses, was repeated with a different enzyme concentration.

The phosphorylase has an abnormally low temperature coefficient. The values for Q_{10} for a temperature range of 20–37° were 0.38, 0.29, and 0.26 for the 30, 60, and 120 minute incubation periods, respectively.⁴ The enzyme which converts the 1- to the 6-ester has a Q_{10} between 2 and 3 and its activity is depressed much more on the acid side than is that of the phosphorylase. Consequently, a low temperature of incubation and an acid pH favor the accumulation of the 1-ester.

1-Ester Inhibition and Effect of Magnesium Ions—The 1-ester has a much stronger inhibitory effect on the rate of phosphoryla-

⁴ Kendal and Stickland (13) claim a Q_{10} of 0 for this temperature range.

tion of glycogen than the 6-ester. This is shown in experiments in which these esters are added to the reaction mixture at the beginning of the incubation period. Owing to the presence of the conversion enzyme which is active even if no Mg^{++} is added (6), the initial 1-ester concentration cannot be maintained. In one such experiment an initial concentration of 7 mM of 1-ester caused an inhibition of 93, 65, and 59 per cent during 10, 20, and

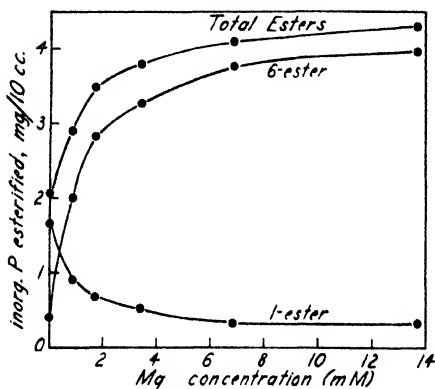


FIG. 4

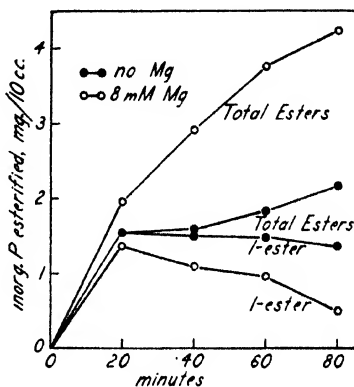


FIG. 5

FIG. 4. Effect of increasing amounts of Mg^{++} on phosphorylation of glycogen. An electrodialyzed muscle extract was incubated for 1 hour at 22° with 0.6 per cent glycogen, $m/30$ phosphate buffer of pH 7.2, 0.8 mM adenylic acid, and varying concentrations of $MgSO_4$. The 1- and 6-ester concentrations were determined separately, their sum representing total ester formation.

FIG. 5. Effect of Mg^{++} at acid pH. A dialyzed muscle extract was incubated at 24.5° with $m/17$ phosphate buffer of pH 6.5, 1 per cent glycogen, and 1 mM adenylic acid. To one set of reaction mixtures was added 8 mM $MgSO_4$, as indicated on the curves.

40 minutes of incubation, respectively, while an initial concentration of 7 mM of 6-ester caused an inhibition of only 17, 15, and 11 per cent. Since the enzymatic conversion of the 1- to the 6-ester is accelerated by Mg^{++} (6), the latter might be expected to have an effect on the rate of phosphorylation of glycogen by preventing the accumulation of the inhibitory 1-ester. This was found to be the case, as shown in Figs. 4 to 6.

In the experiment in Fig. 4 may be seen an inverse relation-

ship between rate of phosphorylation (curve for total esters) and 1-ester concentration. In the experiment in Fig. 5 use is made of the fact that at pH 6.5 the activity of the conversion enzyme is more strongly inhibited than that of the phosphorylating enzyme. Without addition of Mg^{++} the 1-ester concentration reached 5 mM after 20 minutes of incubation and remained at this level for some time; the phosphorylation of glycogen (curve for total esters) practically stopped between 20 and 40 minutes and was only resumed at the end of the incubation period, when a slight decrease in the 1-ester concentration had occurred.

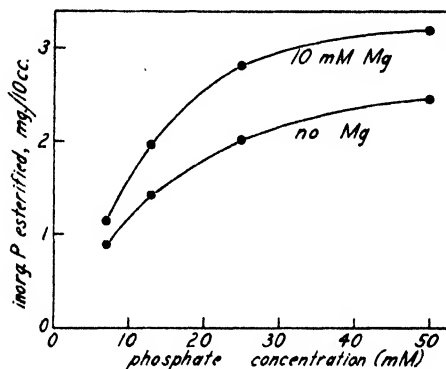


FIG. 6. Effect of Mg^{++} at various phosphate concentrations. A dialyzed muscle extract was incubated for 20 minutes at 37° with 0.6 per cent glycogen, 0.3 mM adenylic acid, and varying concentrations of phosphate buffer of pH 7.2. To one set of reaction mixtures was added 10 mM $MgSO_4$, as marked on the curve.

With addition of Mg^{++} the 1-ester concentration did not reach the same level and decreased more rapidly than without Mg^{++} and the rate of phosphorylation was correspondingly greater. This experiment shows that there exists a critical 1-ester concentration, in this case 5 mM, at which the activity of the phosphorylase is completely inhibited. As this critical concentration is approached, even slight changes in the 1-ester concentration have a marked effect on the rate of phosphorylation.

In the experiment in Fig. 6 the initial phosphate concentration of the reaction mixture varied from 7 mM (which corresponds to that found in resting muscle) to 50 mM (which is nearly

optimal); the effect of Mg^{++} was greater at the high than at the low phosphate concentration. The accelerating effect of Mg^{++} on the phosphorylation of glycogen is attributed to the elimination of 1-ester inhibition. A direct effect of these ions on the phosphorylase seems unlikely.

The optimal concentration of Mg^{++} for the conversion enzyme is 5 mM, that of Mn^{++} and Co^{++} 0.5 mM. These ions may increase the activity of the conversion enzyme 10 to 15 times (6). The effect of these ions, in the above concentrations, on the phosphorylation of glycogen in an electro dialyzed extract (expressed in mg. of inorganic P which disappeared in 1 hour at 37° per 10 cc. of reaction mixture) was as follows: no ions added 5.6, Mg^{++} 10.3, $Mg^{++} + Mn^{++}$ 9.2, Mn^{++} 9.1, Co^{++} 9.0. As in the case of Mg^{++} , the effect of Mn^{++} and Co^{++} on phosphorylation of glycogen is probably an indirect one and is due to greater activity of the enzyme which converts the 1- to the 6-ester.

Glucose in 17 mM concentration inhibited phosphorylation in muscle extract about 50 per cent, while fructose and maltose were without effect. Lehmann (14), who first described the inhibitory action of glucose, discussed its possible physiological significance. However, the glucose concentration of skeletal muscle in the intact animal does not reach very high values. The distribution of glucose between blood and muscle has been investigated on a previous occasion (15). At a glucose level in the plasma of 300 mg. per cent (17 mM), the glucose concentration in muscle was only 50 mg. per cent, at which concentration glucose has no inhibitory effect on phosphorylation.

Effect of Phlorhizin and Other Substances—Lundsgaard (16) discovered that phlorhizin has an inhibitory effect on lactic acid formation in muscle brei and found that it was due to an inhibition of phosphorylation. Ostern *et al.* (17) showed that phlorhizin prevents the formation of 6-ester in muscle extracts incubated with glycogen and inorganic P. The experiments in Table I indicate that phlorhizin inhibits the first step in the breakdown of glycogen, the formation of 1-ester. A concentration of phlorhizin close to 0.01 M is necessary for strong (85 per cent or more) inhibition of phosphorylase activity. This is a concentration far above that reached in the tissues of an intact animal injected with the usual dose of phlorhizin.

The phloretin part of the phlorhizin molecule, when added to muscle extract in 0.01 M concentration, was about as active as phlorhizin, while phloroglucinol had very little inhibitory action. Epinephrine, which accelerates very markedly the formation of 6-ester in intact muscle (18), has no effect on phosphorylation in muscle extract (in a concentration of 1:15,000) and does not overcome the phlorhizin inhibition in muscle extract. Of other substances tested, 0.01 M cyanide, fluoride, and iodoacetate, 0.02 M glyceraldehyde, 0.03 M glutathione, and insulin (10 mg. per cent) had no effect on phosphorylation in muscle extract.

SUMMARY

1. Dialyzed rabbit muscle extracts were incubated with glycogen, inorganic phosphate, $MgCl_2$, and adenylic acid. After 10 to 30 minutes of incubation from 93 to 97 per cent of the glycogen which disappeared was accounted for by the formation of hexosemonophosphate, after longer periods of incubation from 82 to 98 per cent. This close correspondence indicates that the phosphorylase causes a disruption of the entire glycogen molecule into uniform fragments consisting of glucose-1-phosphate. When inorganic P was omitted from the reaction mixture, a small amount of glycogen still disappeared. This could not be attributed with certainty to diastatic activity, because the traces of inorganic P which were not removed by simple dialysis of the extracts sufficed for some ester formation. In an electro-dialyzed and very actively phosphorylating extract no glycogen disappeared when inorganic P was omitted. The phosphorylase therefore disrupts the glycogen molecule without the aid of a diastase.

2. The activity of the enzyme was tested over a 15-fold range of dilutions with 1 per cent glycogen and M/18 phosphate. There was a rapid falling off in the rate of enzyme activity with time at all enzyme concentrations. The time to esterify a given amount of inorganic P was inversely proportional to the enzyme concentration. Some conditions under which such a proportionality did not hold are described.

3. A pH-activity curve showed a broad maximum between pH 7 and 8 with about one-half the activity at pH 5.8 and 9. The temperature coefficient of the enzyme was abnormally low;

between 20° and 37° the Q_{10} was always below 0.5, often as low as 0.25.

4. Glucose-1-phosphoric acid, the first product of glycogen degradation, was found to have a strong inhibitory effect, and its enzymatic conversion product, glucose-6-phosphoric acid, a weak inhibitory effect on the phosphorylase. Glucose was also inhibitory, but fructose and maltose were not. Mg^{++} , Mn^{++} , and Co^{++} , when added to a dialyzed extract, increased the activity of the phosphorylase. Since these ions accelerate the enzymatic conversion of the 1- to the 6-ester, their effect on phosphorylation is attributed to a removal of 1-ester inhibition.

5. Phlorhizin and phloretin in 0.01 M concentration almost completely suppressed the breakdown of glycogen and the formation of 1-ester, while phloroglucinol had little inhibitory action. Cyanide, fluoride, iodoacetate, glyceraldehyde, epinephrine, insulin, and glutathione, in the concentrations tested, were without effect on the phosphorylase.

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